PDZ-Proteins and the Function of the Sodium/Phosphate Cotransporter NaPi-IIa

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SUMMARY

In the kidney, the type IIa Na-P_i cotransporter (NaPi-IIa) plays a key role in brush-border membrane P_i flux. It accounts for at least 70% of the renal phosphate reabsorption. A yeast two hybrid screen, using the C-terminal tail of the NaPi-IIa as bait, identified a number of PDZ domain-containing proteins that interact with NaPi-IIa. Among them, NHERF1 and PDZK1 have been shown to co-localize with NaPi-IIa in the brush border membrane and in the subapical compartment of proximal tubular cells. The interaction of the COOH terminus of NaPi-IIa with NHERF1 and PDZK1 depends on the last three amino acid residues, TRL.

The generation of mouse models deficient for these PDZ proteins has allowed to study the role of NHERF1 and PDZK1 in the localization and regulation of NaPi-IIa *in vivo* and in ex vivo models.

In the first part of this work we investigated the steady state expression of the type IIa sodium phosphate cotransporter, its hormonal regulation, and chronic and rapid adaptation to different diets in *Pdzk1* deficient mice. The localization of NaPi-IIa and other proteins interacting with PDZK1 *in vitro* such as the Na^+/H^+ exchanger (NHE-3) and NHERF1 was not altered in *Pdzk1 -/-* mice. The abundance of NaPi-IIa adapted to acute and chronic changes in dietary P_i intake, but steady-state levels of NaPi-IIa were reduced in *Pdzk1-/-* under a P_i rich diet. This was paralleled by a higher urinary fractional P_i excretion. In contrast, NHERF1 abundance increased in the brush border membrane of *Pdzk1 -/-* mice fed a high P_i diet. Acute regulation of NaPi-IIa by Parathyroid hormone (PTH) *in vivo* and by PTH and activators of protein kinases A, C and G (PKA, PKC and PKG) *in vitro* (kidney slice preparation) was not altered in *Pdzk1* -/- mice. This loss of PDZK1 did not result in major changes in proximal tubule function or NaPi-IIa regulation. However, under a P_i rich diet, loss of PDZK1 reduced NaPi-IIa abundance. Thus PDZK1 may play a role in the trafficking or stability of NaPi-IIa under these conditions.

In the second part, we addressed the role of NHERF1 in the hormonal regulation of NaPi-IIa by examining the PTH induced internalization and signaling pathways in *Nherf1* deficient mice. Immunohistochemistry and Western blotting demonstrated that stimulation of apical and basolateral

receptors with PTH 1-34 led to internalization of NaPi-IIa in wildtype and *Nherf1* deficient mice, while stimulation of only apical receptors with PTH 3-34 failed to induce internalization of NaPi-IIa in NHERF1 Knock-Out mice. Expression and localization of apical PTH receptors were similar in both wildtype and *Nherf1* deficient mice. In wildtype mice, injection of PTH1-34 resulted in acute phosphaturia, whereas in *Nherf1* deficient mice, the phosphaturic response was smaller. In addition, separate activation of the PKC and PKA dependent pathways with DOG or 8-Br-cAMP, respectively, induced normal internalization of NaPi-IIa also in *Nherf1* deficient mice. However, the stimulation of PLC activity via apical PTH receptors was impaired in *Nherf1* deficient mice but normal in wildtype animals. These data suggest that NHERF1 in the proximal tubule is important for proper PTH induced internalization of NaPi-IIa and specifically couples the apical PTH receptor to PLC allowing activation of PLC dependent pathways and subsequent endocytosis of NaPi-IIa.

DEUTSCHE ZUSAMMENFASSUNG DER DOKTORARBEIT

Der Natrium (Na^+) - Phosphat (P_i) Kotransporter (NaPi-IIa) spielt in der Bürstensaummembran der Niere eine wichtigte Rolle für die Phosphatreabsorption aus dem Harn. Dabei trägt NaPi-IIa zu etwa 70% der renalen P_i Reabsorption bei. Die PDZ-Proteine NHERF1 und PDZK1 wurden mittels einer „Yeast-Two-Hybrid“ Untersuchung mit dem C-terminalen Ende von NaPi-IIa als Zielprotein identifiziert und interagieren mit NaPi-IIa *in vitro*. Diese Interaktion mit NHERF1 und PDZK1 benötigt die letzten drei Aminosäuren „TRL“ des Carboxy-terminalen Ende des NaPi-IIa Proteins. Beide Proteine, NHERF1 und PDZK1, kolokalisieren in der Bürstensaummembran und im subapikalen Kompartiment der proximalen Tubuluszellen mit NaPi-IIa. Die genetische Manipulation von Mäusen mit einer Defizienz für diese PDZ-Proteine ermöglichte es nun, die Rolle von NHERF1 und PDZK1 für die Lokalisation und die Regulierung von NaPi-IIa in *in vivo* und *ex vivo* Modellen zu untersuchen.

Im ersten Teil dieser Arbeit untersuchten wir in *Pdzk1*-defizienten Mäusen die Expression von NaPi-IIa, dessen hormonale Regulierung sowie die chronische und akute Adaptation an die Aufnahme von Phosphat mit der Nahrung. Die Lokalisation von NaPi-IIa und anderer Proteine, welche mit PDZK1 *in vitro* interagieren, wie z. Bsp. der Na^+/H^+ Austauscher 3 (NHE-3) oder NHERF1, war in der Niere von *Pdzk1 -/-* Mäusen unverändert. *Pdzk1 -/-* Mäuse konnten sich an akute und chronische Veränderungen der P_i Aufnahme adaptieren. Die NaPi-IIa Expression war jedoch in *Pdzk1 -/-* Mäusen im Vergleich zu Wildtypmäusen unter einer P_i-reichen Diät reduziert. Die Phosphatausscheidung im Urin war übereinstimmed mit diesen Befunden erhöht. Die Expression von NHERF1 in der Bürstensaummembran von *Pdzk1 -/-* Mäusen nahm zu, wenn die Mäuse mit einer stark P_i –haltigen Nahrung gefüttert wurden. Die akute Regulierung von NaPi-IIa *in vivo* durch Parathormon (PTH) oder *in vitro* in Nierenschnitt-Präparationen durch PTH und durch Aktivatoren der Proteinkinasen A, C und G (PKA, PKC, PKG) änderte sich in *Pdzk1 -/-* Mäusen nicht. Zusammenfassend lässt sich sagen, dass ein Fehlen von PDZK1 zu keinen grösseren Veränderungen in der Regulierung von NaPi-IIa führt, jedoch die Expression von NaPi-IIa in

Abwesenheit von PDZK1 unter einer reichhaltigen P_i Diät verringert ist. Folglich könnte PDZK1 unter diesen Bedingungen eine Aufgabe beim Einbau von NaPi-IIa in die Membran oder seiner Stabilität dort haben.

Im zweiten Teil dieser Arbeit befassten wir uns mit der Rolle von NHERF1 in der hormonalen Regulierung von NaPi-IIa durch PTH, wobei die Internalisierung von NaPi-IIa und die Signalkaskade in *Nherf1*-defizienten Mäusen untersucht wurde. Immunhistochemie und Westernblots zeigten, dass die Stimulierung apikaler und basolateraler Rezeptoren durch PTH 1-34 die Endozytose von NaPi-IIa in Wildtyp- und Nherf1-defizienten Mäusen aktivierte, während hingegen die Stimulierung nur apikaler Rezeptoren durch PTH 3-34 keine Internalisierung von NaPi-IIa in *Nherf1*-defizienten Mäusen zur Folge hatte. Die Expression und die Lokalisierung der apikalen PTH-Rezeptoren waren ähnlich in Wildtyp- und Nherf1-defizienten Mäusen. Injektion von PTH 1-34 in Wildtyp-Mäusen resultierte in akuter Phosphaturie, welche aber in *Nherf1*-defizienten Mäusen geringer war. Zudem bewirkte eine getrennte Aktivierung von PKC- und PKA-abhängigen Signalwegen mittels DOG oder 8-Br-cAMP auch eine normale Internalisierung von NaPi-IIa in *Nherf1*-defizienten Mäusen. Jedoch wurde die Stimulierung der Phospholipase C-Aktivität (PLC) via apikale PTH-Rezeptoren in *Nherf1*-defizienten aber nicht in Wildtyp-Mäusen unterdrückt. Diese Resultate deuten darauf hin, dass NHERF1 im proximalen Tubulus zur korrekten PTH-induzierten Internalisierung von NaPi-IIa wichtig ist und dabei NHERF1 speziell den apikalen PTH-Rezeptor an PLC koppelt, was eine Aktivierung von PLC-abhängigen Signalwegen und die weiterführende Endozytose von NaPi-IIa erlaubt.

1 INTRODUCTION

Inorganic phosphate (P_i) is the sixth most abundant element in the human body. It is an important compound for cellular function, as it is a constituent of metabolic intermediates, lipids, nucleic acids, “energy-storing” molecules such as ATP, and proteins [29]. It is also fundamental to skeletal mineralization.

The required phosphate is supplied by the daily diet. Normal phosphate intake in the adult is in the range of 800-1600 mg/day. About 65 to 75 % of the ingested phosphate is absorbed in the small intestine, is released into blood, deposited in organs or is finally filtered in the glomeruli of the kidney.

In blood, phosphate exists in two forms: in an organic form, mainly as phospholipids and phosphate esters, and in an inorganic form, which accounts for ~29 % of the total plasma phosphate. Of this 10-15 % is bound to proteins and the remaining can be freely filtered in the renal glomeruli. At the physiological blood pH 7.4 the main form is the divalent phosphate anion HPO_4^{2-}.

In the body, approximately 85 % of the total phosphate is stored in the skeleton and teeth, whereas the remaining is distributed in soft tissues (14 %) and in extracellular fluids (1 %). Extracellular phosphate in adults is constantly kept in a narrow range between 0.8 - 1.5 mM. This is achieved mainly by a control of the renal capacity to reabsorb phosphate from the primary urine and to a lesser extent by the control of absorption of dietary phosphate in the small intestine. Indeed, in contrast to intestinal absorption, which is adjusted rather slowly, renal excretion can be adjusted rather rapidly to altered physiological conditions. Thus, renal phosphate excretion is the balance between free glomerular filtration and regulated tubular reabsorption. Under physiological condition ~80 - 90% of filtered load is reabsorbed along the renal proximal tubules (for review see [29, 33, 43]).

The cellular mechanisms involved in the proximal tubular P_i reabsorption have been studied by a variety of techniques, including *in vivo* and *in vitro* microperfusion, tissue culture techniques and studies with isolated brush-border and basolateral membrane vesicles. From these studies a secondary

active transport scheme emerged. Phosphate is taken up from the tubular fluid by brush-border membrane sodium-phosphate cotransporters and leaves the cell via basolateral transport pathways. The brush border entry step is the rate limiting step and the target for almost all physiological and pathological mechanisms altering phosphate reabsorption. The basolateral exit pathway is not well defined. Several membrane proteins have been identified that transport phosphate in a sodium dependent manner. Among those, NaPi-IIa appears to be of crucial importance for overall renal phosphate reabsorption [32].

In the past few years a complex network of PDZ interactions involved in the apical expression of membrane proteins in epithelial cells has emerged. Much of this work has been done in renal proximal tubular cells, airway and intestinal cells and has mostly focused on the effect of PDZ proteins on the expression and function of NHE-3, CFTR and NaPi-IIa [7].

The generation of mouse models deficient for some of these PDZ proteins [22, 42] has made it possible to study *in vivo* and *in vitro* the role of these PDZ-based interactions on the localization and regulation of transport proteins such as NaPi-IIa.

2 SEVERAL FAMILIES OF SODIUM DEPENDENT PHOSPHATE TRANSPORTERS

Several sodium-dependent phosphate cotransporter have been identified on a molecular level. They are classified, according to the Human Gene Nomenclature Committee Database (http://gene.ucl.ac.uk/nomenclature/) in the large superfamily of Solute Carriers (SLC) [33]:

2.1 Type I NaPi Cotransporters (SLC17)

The type I NaPi cotransporter is localized in the proximal tubular brush border membrane, with higher expression in deep juxtamedullary nephrons than in superficial nephrons. The protein may contain 6 to 8 transmembrane domains; it contains 3 N-glycosylation motifs; the induced phosphate transport activity in Xenopus oocytes does not show any pH dependency. From electrophysiological studies in oocytes, evidence was obtained that the type I transporter protein might be a multifunctional transporter for different organic anions rather than a specific phosphate transporter [33]. The type I NaPi is not regulated by factors known to affect renal phosphate handling.

2.2 Type II NaPi Cotransporters (SLC34)

The SLC34 family comprises three members: NaPi-IIa (SLC34A1), NaPi-IIb (SLC34A2), and NaPi-IIc (SLC34A3) [31]. Type II NaPi cotransporters are expressed in several epithelial tissues and play a major role in phosphate homeostasis. In kidney and small intestine, two important sites that control the extracellular concentration of phosphate, type II NaPi cotransporters are located at the apical site of the epithelial cells and represent the rate limiting step for transepithelial movement of phosphate. In both tissues the

abundance of the type II NaPi cotransporters is controlled by many hormones and metabolic factors, according to the body's phosphate needs. Besides the kidney, expression of NaPi-IIa has been described in bone cells and neurons. Expression of NaPi-IIb has been described in small intestine and other organs, such as lung, liver, and lactating mammary glands. Expression of NaPi-IIc is found exclusively in kidney and has been described as growth related [39]. The protein is localized in apical membranes of proximal tubules of deep nephrons.

In a physiological environment, all SLC34 family members exclusively transport phosphate ions in an obligatory sodium-dependent manner and, with the exception of NaPi-IIc, NaPi cotransport is electrogenic.

2.3 Type III NaPi Cotransporters (SLC20)

The Receptor for Gibbon Ape Leukaemia Virus (Glvr-1) and the Receptor for the Mouse Amphotropic Retrovirus (Ram-1) have been shown to mediate Na-P_i transport when heterologously expressed in oocytes. They have been renamed PiT-1 and PiT-2. Their mRNA is almost ubiquitously expressed; their protein localization has not been elucidated to date. In mouse proximal tubule they might be located on the basolateral membrane and play a role in house-keeping of cellular phosphate homeostasis. Their expression seems not to be altered by PTH. They show a 3:1 stoichiometry, reflecting an electrogenic transport activity [33].

3 THE SODIUM-PHOSPHATE COTRANSPORTER NaPi-IIa

The type IIa NaPi cotransporter (SLC34A1) plays a key role in brush-border membrane P_i flux. Changes in its expression correspond to alterations in proximal tubular phosphate handling. In addition, the disruption of the *Npt2* gene in mice leads to a reduction by 70% of the brush border NaPi cotransport rate documenting its physiological importance [5]. The remaining activity may be due to the type IIc cotransporter [39].

3.1 General Characteristics Of The Sodium Phosphate Cotransporter Type IIa

The type IIa NaPi cotransporter is preferentially expressed in the kidney, with an apical proximal tubular location, but also in bones, where it may be involved in phosphate release from bone resorption. Under normal physiological conditions, the abundance of NaPi-IIa is highest in S1 proximal tubular segments (from the glomerulus to the first part of the proximal convoluted tubule) of juxtamedullary nephrons [27]. The type IIa NaPi cotransporter has eight transmembrane domains; both N- and C- termini are located cytoplasmatically [5]; multiple glycosylation sites were identified in a large extracellular loop; two regions (intracellular loop 1 and extracellular loop 3) have been show to be critical for the transport process (Fig.1). NaPi-IIa contains numerous potential phosphorylation sites, but whether they can play a role in the regulation of the transporter is not yet clear.

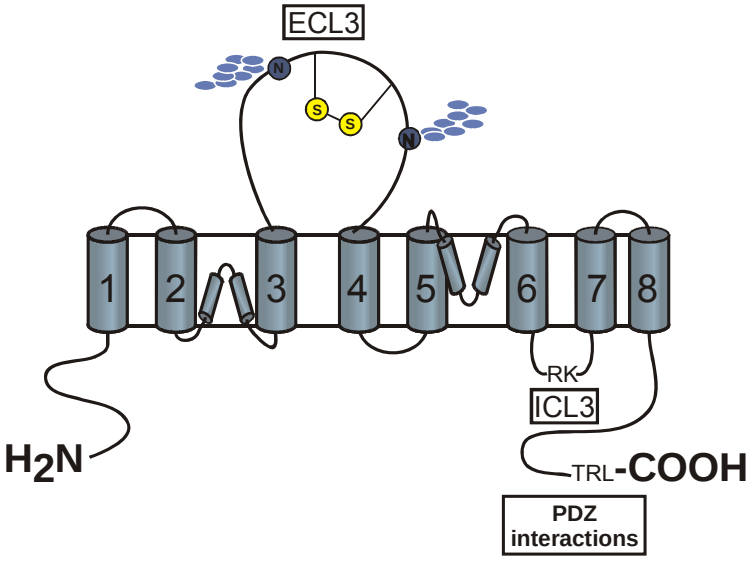

Fig. 1 – Predicted structure of NaPi-IIa

When expressed in oocytes, the type IIa NaPi cotransporter

mediates sodium-phosphate cotransport activity with functional characteristics identical to those observed in isolated brush border vesicles. It shows a $3Na^+$:$1P_i$ stoichiometry leading to an electrogenic transport activity with the inward transfer of one net positive charge per transport cycle. Clear pH dependence has been demonstrated for the type IIa NaPi cotransporter, which is partly due to the preference for the divalent HPO_4^{2-} form, but also due to a competition of protons with sodium ions for specific binding sites [12]. This pH dependence is determined by basic amino acid residues (REK) in the third extracellular loop (ECL3, Fig.1) [10]. The substrates (three Na^+ ions and one HPO_4^{2-} ion) bind in an ordered manner: $Na/P_i/2Na$. Typical apparent substrate affinities are: K_m^{Pi} ~ 0.1 mM, K_m^{Na} ~ 70 mM. On immunoblots of brush border membrane proteins performed under non reducing conditions, the type IIa shows an apparent molecular weight of 80 - 90 kDa; under reducing conditions two bands of 45 - 50 kDa appear. The type IIa protein can homomultimerize, but the functional unit is most likely a monomer [23]. It can also interact with other proteins in the brush border and in the subapical compartment (*see Interacting Proteins*, [16]).

3.2 Regulation of the Type IIa NaPi Cotransporter

Urinary phosphate excretion is subjected to a tight regulation to fulfill the "homeostatic" function by keeping the extracellular phosphate concentration in a narrow range (1.1 mM in adults). This occurs mainly via the control of the amount of NaPi-IIa cotransporter molecules residing in the brush border membrane by regulated endocytosis and posttranscriptional events. Internalized NaPi-IIa proteins do not recycle, but undergo lysosomal degradation (for review see [29, 33, 43]).

The NaPi-IIa cotransporter protein is tightly regulated by different factors such as dietary P_i intake and hormones (see below).

Furthermore, several phosphate wasting disorders have been described that affect the expression of NaPi-IIa, such as the inherited disorders X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), and the acquired disorder oncogenic hypophosphatemic

osteomalacia (OHO). Studies on these disorders have revealed evidence that the two genes PHEX and FGF23 (fibroblast growth factor 23) play an important role in determining the abundance of NaPi-IIa in renal proximal tubules. With respect to FGF23, it has been shown that this protein may be involved in dietary regulation of proximal tubular phosphate reabsorption, independently of changes in the plasma concentration of other phosphaturic hormones such as PTH [40].

3.2.1 Dietary Phosphate Adaptation

Dietary phosphate adaptation is (partly) independent from other factors such as Parathyroid hormone (PTH). *In vivo* and cell culture studies indicate that adaptation to chronic phosphate restriction (days or weeks) depends on *de novo* protein synthesis. In contrast, the fast (2-4 h) adaptive response to acute phosphate deprivation does not seem to be dependent on *de novo* protein synthesis, but rather on the translocation of a subapical pool of NaPi-IIa proteins into the brush border membrane. Similarly, in the acute adaptation to a high-P_i diet/containing medium the decreased rate of Na^+/P_i cotransport is associated with a decrease in NaPi-IIa protein but not NaPi-IIa mRNA abundance [26, 35].

3.2.2 Hormonal Regulation

The major hormone playing a role in the regulatory machinery of the NaPi-IIa protein is the Parathyroid Hormone (PTH). Its action leads to urinary phosphate wasting, as shown by parathyroidectomy or by PTH injections in mice and rats [36, 37] and primary hyperthyroidism in man. Furthermore, shortly after PTH injection, the amount of NaPi-IIa in the brush border membrane is strongly decreased. NaPi-IIa proteins are in fact internalized via the endocytic pathway of receptor-mediated endocytosis. Internalization occurs at the microvillar cleft via clathrin-coated vesicles [4]. In the kidney, PTH interacts with a G-protein-coupled receptor (PTH1R) expressed both in

the apical and in the basolateral membrane of the proximal tubule cells. It has been shown that in proximal tubular cells, treated with either the synthetic PTH fragment 1-34 or the fragment 3-34 it is possible to detect a strong as well as rapid down-regulation of NaPi-IIa due to the retrieval of the protein form the brush border membrane and its subsequent routing to the lysosomes for degradation. Furthermore evidence has been provided that, although PTH 1-34 is active from both apical and basolateral side, PTH 3-34 is only effective when it interacts with the apically located receptor. The action of PTH 1-34 seems to activate both PKA and PKC-dependent pathways, whereas PTH 3-34 is only able to activate the PKC pathway [44].

A dibasic amino acid motif (RK) within the putative intracellular loop ICL3 (Fig. 1) has been shown to be important for the PTH mediated endocytosis of the transporter, as, after mutation of this site, NaPi-IIa is no longer responsive to the hormone [20]. This motif may interact with PEX19, a protein involved in binding and trafficking of peroxisomal proteins [18].

Recently, the involvement of other signaling pathways, such as PKG and ERK1/2-MAPK-dependent pathways, has been reported [2, 3].

The events after second messenger formation leading to inhibition and/or retrieval of NaPi-IIa are not clear. Phosphorylation events may play a role, but there is no evidence that altered phosphorylation of the transporter itself occurs or is essential for regulation. It is reasonable to assume that PTH-induced regulatory phosphorylation events are not at the level of the transporter but rather at the level of interacting proteins required for its regulation. Indeed, PTH induced phosphorylation of NHERF1, interacting with NaPi-IIa (see below), was recently shown [11].

3.2.3 Interacting Proteins

The microvillar localization of the NaPi-IIa protein and its physiologically controlled abundance in the brush border membrane suggest the existence of specific interactions with other microvillar/subapical proteins. These “interacting” proteins might be involved in the anchoring of the transporter into the membrane and/or be required for the correct organization of the signaling

complexes that lead to internalization of the protein. Indeed, PDZ domain-containing proteins have been described to interact with NHE-3 and to be required for its kinase mediated regulation and internalization [47]. To identify such candidate proteins for the interaction with the NaPi-IIa cotransporter, a yeast two hybrid screen has been performed, using the C-terminal tail of the NaPi-IIa cotransporter as bait allowing the identification of a number of cytosolic PDZ domain-containing proteins [16]. Some of these have been shown to co-localize with NaPi-IIa at the brush border and in the subapical compartment of proximal tubular cells, such as NHERF1 and PDZK1. The interaction of the COOH terminus of NaPi-IIa with NHERF 1 and PDZK1 depends on the last three amino acid residues, TRL, which represent a class 1 PDZ-binding domain. Although PDZK1 contains four and NHERF1 two PDZ domains, the interaction with NaPi-IIa occurs specifically with PDZ-3 of PDZK1 and with PDZ-1 of NHERF1. Recently, a new yeast two-hybrid technology based on split-ubiquitin, which allows studying interactions of full length and membrane inserted proteins, has confirmed these earlier findings and demonstrated that full length and membrane inserted NaPi-IIa interacts with NHERF1 and PDZK1 via the COOH terminal PDZ binding domain TRL [13].

4 PDZ PROTEINS

PDZ domains are ubiquitous elements of cytoplasmic proteins in organisms from bacteria to mammals. Due to a common multiple copy occurrence within a single protein they mediate formation of extensive protein-protein networks. Diversity and size of such protein complexes is further enhanced by combination of PDZ domains with other protein interaction modules (SH3, PTB, LIM, WW and ankiryn repeats) (for review see [6, 7, 19, 34].

Among major cellular targets of PDZ domains are proteins directly associated with the plasma membrane like ion channels, receptors and cytoskeleton proteins. The structural basis of their specificity to bind four to six C-terminal residues of these proteins appears relatively simple and suggests redundancy of recognized target sequences. However, PDZ domains can also bind other PDZ domains in a head-to-tail fashion, recognizing internal structural motifs in their target proteins and bind to phosphatidylinositol derivates [19].

PDZ domains derive their name from the proteins in which they were first discovered: Postsynaptic density 95 (PSD-95), disc large (Dlg), and zonula occludens-1 (ZO-1). They are also known as DHRs (Disc-Large Homology Regions) or GLGF repeats (after the highly conserved four-residue motif within the domain).

4.1 PDZ Domain Classes

PDZ domains are 80 to 100 amino acids long sequences arranged in a globular structure, specialized for binding of carboxy-terminal sequences of proteins. PDZ domains can also bind internal peptide sequences that structurally mimic the C-terminus.

PDZ domains specifically recognize short (typically about five residues long) carboxy-terminal peptide motifs. On the basis of the binding specificity (target sequence), three classes of PDZ domains have been described:

PDZ domain class	TARGET sequence
I	X-S or T-X-V or L
II	XΨXΨ
III	X-D or E-X-Ψ

X = any amino acid

Ψ = hydrophobic residue

Class I – The first class group PDZ domains having the consensus sequence X-S or T-X-V or L (any- Ser or Thr-any-Val or Leu) in the target protein. Examples of proteins that recognize such PDZ domains are NHERF1 and NHERF2 and PDZK1 (n.b. NHE-3, NaPi-IIa and CFTR contain prototypical COOH-terminal class I PDZ domains: STHM for NHE-3, ATRL for NaPi-IIa and DTRL for CFTR)

Class II – The recognized sequence is: XΨXΨ (Ψ = hydrophobic residue, such as Val, Tyr, Phe, Leu, Ile) and it is recognized by proteins like CASK, which is a calcium/calmodulin-dependent serine kinase localized in synapses where, as a scaffold, it is responsible for trafficking of NMDA receptors to the plasma membrane.

Class III – X-D or E-X-Ψ (any-Asp or Glu-any-Val). The neuronal nitric oxide synthase nNOS binds to such a PDZ domain.

4.2 PDZ Domain-containing Proteins

PDZ proteins can be classified according to their molecular organization:

1. The first family consists of proteins containing only PDZ domains, the number of which can vary from two (as in NHERF1 and 2) to more than ten.

2. The second family comprises the MAGUKs (membrane associated guanylate kinases, including PSD-95, Dlg and ZO-1) that contain up to three PDZ domains, one SH3 domain and a guanylate kinase domain.

3. The third family encompasses proteins that contain PDZ domains together with other protein-interacting domains, such as ankyrin repeats, LIM, L27, C2, PH, WW, DEP and LRR domains.

In most cases the binding to a PDZ domain is constitutive, but sometimes an agonist-dependent activation is required (i.e. NHERF1 only interacts with the activated β2-adrenergic receptor [17]). Sometimes, PDZ interactions are disrupted by phosphorylation of the PDZ-binding site, although it has been demonstrated that phosphorylation can also increase the strength of the interaction.

Generally, PDZ proteins are key elements in building functional protein complexes. They are indeed involved in the scaffolding of the appropriate localization of a number of proteins at the apical or the basolateral membrane of polarized epithelia or neurons; they provide a backbone for the spatial arrangement of receptors and other components for a correct signal transduction process and are involved in the regulatory machinery of a number of membrane proteins.

4.2.1 NHERF1

Na^+/H^+ exchanger regulatory factors (NHERF1 and 2) are PDZ proteins that associate with the apical brush-border membrane in polarized epithelial cells. NHERF1, also known as EBP50 (ezrin-radixin-moesin binding phosphoprotein-50) was originally cloned as an essential cofactor for the protein kinase A (PKA)-mediated inhibition of the Na^+/H^+ exchanger isoform 3 (NHE-3) in renal proximal tubule cells [48]. NHERF2, also known as E3KARP, is a structurally and functionally related isoform [49]. In mouse proximal tubule cells, NHERF1 is strongly expressed in microvilli, whereas NHERF2 is predominantly expressed at the microvillar basis in the vesicle rich domain [45]. NHERF1 and 2 contain two tandem PDZ domains that can bind to a long list of ion channels (i.e. CFTR, ROMK K^+ channels), ion transporters (NHE-3, NaPi-IIa), and receptors (β_2-adrenergic receptor) with a relative degree of specificity for binding to the first or the second PDZ domain [41]. NHERF proteins also contain at their COOH terminus an ezrin-radixin-moesin (ERM)-binding domain that interacts with ezrin, a scaffolding protein associated with the actin cytoskeleton [30]. In addition, ezrin functions as an A kinase anchoring protein (AKAP): it interacts with the regulatory subunit (RII) of PKA,

thereby recruiting PKA to the PDZ complex. The multivalent properties of NHERF (PDZ1, PDZ2, ERM-binding domain) allow the formation of macromolecular complexes that anchor membrane proteins to the apical actin cytoskeleton. Typical examples include the NHE-3-NHERF1-ezrin [24] and NaPi-IIa-NHERF1-ezrin [25] complexes attached to the actin cytoskeleton in the microvilli of proximal tubule cells in the kidney. Finally, although NHERF1 and 2 share overlapping expression and binding profiles, they fulfill unique properties with respect to various membrane proteins, possibly due to their partially different subcellular localizations.

Because NHERF1 and 2 are multivalent scaffolds, they can assemble functionally connected proteins (receptors, kinases, transporters) into a spatially restricted unit (so-called transducisome), which allows tight control and efficient activation/ inhibition of membrane transport processes [41]. For example, the basal Na^+/H^+ exchange activity in brush-border membranes of renal proximal tubule cells is indiscernible between wildtype and *Nherf1* deficient mice [42]. However, the cAMP-dependent inhibition of NHE-3 and the concomitant cAMP-stimulated phosphorylation of NHE-3 are observed only in NHERF1 expressing preparations [9, 47]. Thus one function of NHERF-based protein complexes maybe the formation of multiprotein signaling complexes (transducisomes) in apical membranes.

NHERF1 and 2 proteins also affect membrane expression and localization of apical proteins. This is most clearly illustrated for NaPi-IIa, which normally resides in the apical brush border membrane of renal proximal tubule cells. However, in kidneys from *Nherf1* deficient (-/-) mice, NaPi-IIa is redistributed to intracellular vesicles, thereby reducing NaPi-IIa expression in the apical membrane. Consistent with the altered NaPi-IIa localization, *Nherf1* -/- mice display a mild renal phosphate wasting phenotype [42]. Interestingly, NHERF1 is not required for apical localization of NHE-3 in the proximal tubule, because NHE-3 was expressed at normal levels in the apical brush-border membrane of *Nherf1* -/- kidneys [42]. These data indicate that NHERF1 in epithelial cells plays a crucial role in the apical positioning of some (but clearly not all) PDZ ligand-containing membrane proteins.

4.2.2 PDZK1

PDZK1 (previously also called CAP70 or NaPi-Cap1) is a PDZ-only protein containing four PDZ domains and is expressed in kidney, intestine, and liver [21]. Renal expression of PDZK1 is restricted to the proximal tubule, where it localizes to the apical brush-border membrane (microvilli and intermicrovillar clefts). PDZK1 apical positioning in the proximal tubule like cell line OK requires the interaction with other proteins such as MAP17, an apical membrane protein that forms a PDZ-dependent complex with PDZK1 [38]. In proximal tubule cells, PDZK1 and NHERF1 form an extended network underneath the brush-border membrane that may serve as a docking station for apical proteins. Because of its four PDZ domains, PDZK1 can simultaneously bind several membrane proteins via their COOH-terminal PDZ ligands: Npt2 (NaPi-IIa), the solute carrier SLC17A1 (NaPi-I), NHE-3, the organic cation transporter (OCTN1), the Cl^- formate exchanger (CFEX), and the urate anion exchanger (URAT1). PDZK1 forms heteromeric complexes with NHERF1, which possibly anchors the network to the apical cytoskeleton. In addition, the PDZK1/NHERF1 scaffold recruits several types of signaling proteins. G protein-coupled receptors, such as the purinergic P2Y1 receptor or the parathyroid hormone I receptor (PTHIR) receptor, bind to NHERF via a PDZ-based interaction. Furthermore, PKA is positioned in the apical network via various AKAPs such as ezrin and D-AKAP2, which bind to PDZK1 [14, 15]. So, PDZK1, in combination with NHERF, may establish an extensive network of transport and signaling proteins beneath the brush-border membrane of renal proximal tubule cells. Finally, in hepatocytes the expression of the high-density lipoprotein (HDL) receptor scavenger receptor class B type I (SR-BI) critically depends on PDZK1. *Pdzk1 -/-* mice show strongly reduced levels of hepatic SR-BI and consequently have increased plasma levels of high-density lipoprotein particles [22].

5 MOUSE MODELS USED IN THIS STUDY

5.1 PDZK1 Knock-Out Mice

The *Pdzk1* gene was deleted by homologous recombination and the mice were observed for more than a year [22]. They showed a normal growth profile with no alterations in the gross morphology, weight, size and fertility, as well as no change in the histology of multiple organs, compared to the wildtype (WT) littermates. *Pdzk1 -/-* mice do not show redistribution of interacting proteins or alterations in their mRNA content. Chemistry profiles of serum and urine from these mice were compared to those from wildtype and the only notably difference was found in the serum levels of cholesterol, suggesting a possible role of PDZK1 in its metabolism or handling. The lack of major changes leads to the conclusion that a compensatory mechanism might take place in the absence of this scaffolding protein, particularly concerning the renal environment.

5.2 Nherf1 deficient Mice

Weinman and colleagues generated by homologous recombination a mouse lacking the expression of the *Nherf1* gene [42]. About 75% of the *Nherf -/-* females were generally smaller than their wildtype and heterozygous littermates and showed impaired mobility and a reduced bone mineral density. Most of them died 30-35 days after birth. All *Nherf -/-* male and 25% of the female resembled their wildtype and heterozygous littermates in body size and were fertile. Histological analyses of kidney, brain, liver, spleen and pancreas, from wildtype and *Nherf1* null mice showed similar morphology.

The only notably difference between wildtype and Nherf -/- mice was that *Nherf1* null mice showed a statistically lower serum phosphate concentration and a ~3 fold increase in urinary phosphate excretion, despite an overall normal renal function (urine output and creatinine clearance were normal).

This suggested a defect in renal tubular reabsorption of phosphate. Indeed, immunoblots of renal proximal tubular brush border membrane fractions from wildtype and *Nherf1 -/-* mice, showed a ~50% reduction in the NaPi-IIa protein amount in null mice. This was also confirmed by immunohistochemistry, showing a more subapical localization for the transporter in *Nherf1 -/-* kidney compared to wildtype.

6 AIM OF THE WORK

The sodium phosphate cotransporter type IIa (NaPi-IIa), located in the brush border membrane of the proximal tubular cells, represents the major renal phosphate absorptive mechanism. Its activity is tightly regulated by dietary intake of phosphate, acid-base status, and several hormones including PTH. In order to identify interacting proteins that may be involved in its regulation and membrane localization, we performed previously a yeast-two-hybrid screen with the C-terminus of mouse NaPi-IIa that contains the TRL binding consensus sequence. NHERF1 and PDZK1 were identified as two proteins that interact in several in-vitro assays and colocalized in the proximal tubular brush border membrane.

The generation of mouse models deficient for some of these PDZ proteins has made it possible to study the role of these PDZ-based interactions on the localization and regulation of transport proteins, such as NaPi-IIa.

Thus, the aim of this study was to investigate:

1. The steady state expression of the sodium phosphate cotransporter type IIa, its hormonal regulation and chronic and rapid adaptation to different diets in *Pdzk1* deficient mice
2. The role of NHERF1 in the hormonal regulation of NaPi-IIa by PTH examining the PTH induced internalization and signaling pathways in *Nherf1* deficient mice.

7 PUBLICATIONS THAT CONTRIBUTED TO THIS WORK

7.1 Expression and regulation of the renal Na/Phosphate cotransporter NaPi-IIa in a mouse model deficient for the PDZ protein PDZK1

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EPITHELIAL TRANSPORT

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Expression and regulation of the renal Na/phosphate cotransporter NaPi-IIa in a mouse model deficient for the PDZ protein PDZK1

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Abstract Inorganic phosphate (P_i) is reabsorbed in the renal proximal tubule mainly via the type-IIa sodium-phosphate cotransporter (NaPi-IIa). This protein is regulated tightly by different factors, among them dietary P_i intake and parathyroid hormone (PTH). A number of PDZ-domain-containing proteins have been shown to interact with NaPi-IIa in vitro, such as Na^+/H^+ exchanger-3 regulatory factor-1 (NHERF1) and PDZK1. PDZK1 is highly abundant in kidney and co-localizes with NaPi-IIa in the brush border membrane of proximal tubules. Recently, a knock-out mouse model for PDZK1 ($Pdzk1^{-/-}$) has been generated, allowing the role of PDZK1 in the expression and regulation of the NaPi-IIa cotransporter to be examined in in vivo and in ex vivo preparations. The localization of NaPi-IIa and other proteins interacting with PDZK1 in vitro [Na^+/H^+ exchanger (NHE3), chloride-formate exchanger (CFEX)/putative anion transporter-1 (PAT1), NHERF1] was not altered in $Pdzk1^{-/-}$ mice. The abundance of NaPi-IIa adapted to acute and chronic changes in dietary P_i intake, but steady-state levels of NaPi-IIa were reduced in $Pdzk1^{-/-}$ under a P_i rich diet. This was paralleled by a higher urinary fractional P_i excretion. The abundance of the anion exchanger CFEX/PAT1 (SLC26A6) was also reduced. In contrast, NHERF1 abundance increased in the brush border membrane of $Pdzk1^{-/-}$ mice fed a high-P_i diet. Acute regulation of NaPi-IIa by PTH in vivo and by PTH and activators of protein kinases A, C and G (PKA, PKC and PKG) in vitro (kidney slice preparation) was not altered in $Pdzk1^{-/-}$ mice. In conclusion, loss of PDZK1 did not result in major changes in proximal tubule function or NaPi-IIa regulation. However, under a P_i-rich diet, loss of PDZK1 reduced NaPi-IIa abundance indicating that PDZK1 may play a role in the trafficking or stability of NaPi-IIa under these conditions.

Keywords PDZ proteins · Proximal tubule · Phosphate transport · Brush border membrane · NHERF · PDZK1 · Mouse

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Introduction

Inorganic phosphate (P_i) reabsorption in renal proximal tubules is mediated mainly by the type-IIa sodium-phosphate cotransporter (NaPi-IIa) [20, 21, 23]. This protein is regulated tightly by different factors such as dietary P_i intake, systemic acid-base status and hormones [20]. Among these parathyroid hormone (PTH) plays an important role, increasing the rate of endocytosis of the cotransporter via activation of a complex signalling network involving different protein kinases such as PKA and PKC [22].

In a recent yeast two-hybrid screen we have identified several proteins interacting with the C-terminal TRL motif of NaPi-IIa [8]. These proteins include Na^+/H^+ exchanger-3 regulating factor-1 (NHERF1) and PDZK1 (formerly mouse NaPi-Cap1) that contain multiple PDZ domains. The interaction between NaPi-IIa and PDZK1 and NHERF1 requires the PDZ-binding motif of NaPi-IIa and maps to the PDZ domain 1 of NHERF1 and PDZ domain 3 of PDZK1 [8, 10]. In addition, the interactions have been confirmed further in in vitro assays such as overlays, pull-downs and co-immuno-precipitations from native tissue as well as from cells [8, 9, 10, 26].

The role of NHERF1 as an important protein regulating NaPi-IIa function in the proximal tubule has emerged from several lines of evidence. Disruption of the interaction between the C-terminal TRL motif of NaPi-IIa and PDZ domain 1 in opossum kidney (OK) cells results in loss of apical positioning of NaPi-IIa [11]. Similarly, apical expression of NaPi-IIa in the brush border membrane is reduced in NHERF1-deficient mice [25]. In addition, loss of NHERF1 seems to disrupt the normal adaptive up-regulation of NaPi-IIa with a low dietary P_i intake or after switching the culture medium to a low P_i content [5, 28]. In addition, PTH-induced down-regulation of NaPi-IIa is altered in primary cell culture [5] or in freshly isolated kidney slices derived from NHERF1-deficient mice [27]. Thus, NHERF1 appears to play a role in positioning and regulating NaPi-IIa.

PDZK1 was originally identified as interacting with the membrane-associated protein of 17 kDa (MAP17) in a yeast two-hybrid screen [14]. The same method also showed interaction of PDZK1 with NaPi-IIa and, via its four PDZ domains, also with other proteins expressed in the proximal tubule such as NHE3, chloride-formate exchanger/putative anion transporter-1 (CFEX/PAT1; SLC26A6), the organic cation transporter OCTN1, the urate transporter URAT1, NHERF1, the protein kinase A anchoring protein D-AKAP2, and MAP17, a protein of unknown function [9, 10, 24]. The role of PDZK1 in the proximal tubule has remained unclear due to the lack of appropriate cell culture models. However, it has been suggested that PDZK1 could form, together with NHERF1 and other scaffolding proteins, a network of proteins involved in the trafficking, apical positioning and regulation of various transporters, receptors, other components of signalling cascades and proteins mediating endo- or exocytosis of these complexes [9, 10, 12].

The recent generation of a PDZK1-deficient mouse model has allowed us to study the role of PDZK1 in the expression, localization and regulation of the NaPi-IIa cotransporter in in vivo and in ex vivo preparations [15]. This study showed that loss of PDZK1 did not result in major changes in proximal tubule function and NaPi-IIa regulation. However, under a P_i-rich diet, loss of PDZK1 reduced NaPi-IIa abundance and increased fractional P_i excretion, indicating that PDZK1 may play a role in the trafficking or stability of NaPi-IIa under these conditions.

Materials and methods

Animal studies

Experiments were performed with age- and sex-matched wild-type mice 129SV/EV (*Pdzk1*$^{+/+}$) and *Pdzk1* knock-out mice (*Pdzk1*$^{-/-}$) with the same genetic background, weighing 30–35 g at 22–24 weeks of age. The generation, breeding, and genotyping of these mice has been described previously [15, 16]. Animals were housed in climatized animal facilities and received diets (Kliba, NAFAG, Switzerland) with a high (1.2%) or low (0.1%) P_i content and had free access to water. For some experiments mice were trained to receive food for only 1 h daily to time food intake.

Spontaneous urine samples were collected daily at the same time and frozen rapidly until further analysis. Blood samples were collected immediately before sacrificing the mice by puncture of the vena cava. All samples were analysed for P_i and creatinine using commercial kits (Sigma Diagnostics, St. Louis, Mo., USA and Wako Chemicals, Neuss, Germany) according to the manufacturers' protocols. All animal studies were approved by Harvard Medical School Animal Care Committee.

Western blot analysis

Mice were anaesthetized with ketamine-xylazine i.p., perfused through the left ventricle with warm (37 °C) sucrose/phosphate buffer (140 mM sucrose, 140 mM NaH_2PO_4/NaH_2PO_4, pH 7.4) and the kidneys removed rapidly and frozen until further analysis. Frozen kidneys were used for brush border membranes preparation as described previously using the Mg^{2+}-precipitation technique [4].

For Western blots, brush border membrane protein concentration was measured (Biorad Protein kit) and 10 μg protein solubilized in Laemmli sample buffer containing 2% (v/v) 2-mercaptoethanol. SDS-PAGE was performed on 10% polyacrylamide gels. Proteins were transferred electrophoretically from gels to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, Mass., USA). After blocking with 5% milk powder in TRIS-buffered saline containing 0.1% Tween-20 for 60 min, the blots were incubated with the primary antibodies (rabbit anti-PDZK1 [8] 1:500, rabbit anti-NaPi-IIa 1:6,000 [6], mouse monoclonal anti-actin (Sigma), rabbit anti-NHERF1/2 (kindly provided by E. Weinman, University of Maryland) 1:4,000, rabbit anti-CFEX (kindly provided by P.S. Aronson, Yale University [13]) 1:1,000, rabbit anti-MAP17 [24] 1:4,000), rabbit anti-NHE3 (kindly provided by O.W. Moe, University of Texas, Dallas) 1:5,000 overnight at 4 °C or 2 h at room temperature. After washing and subsequent blocking, blots were incubated with the secondary antibodies (donkey anti-rabbit 1:10,000 or sheep anti-mouse 1:10,000, respectively) IgG-conjugated with horseradish peroxidase (Amersham Life Sciences) or IgG-conjugated with alkaline phosphatase (Promega, Madison, Wisc., USA) for 1 h at room temperature. Antibody binding was detected with the peroxidase/luminal enhancer kit (Pierce, Rockford, Ill., USA) or with CDP-Star (Roche Diagnostics, Indianapolis, Ind., USA) respectively, using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analysed using appropriate

software [Advanced Image Data Analyser (AIDA), Raytest] to calculate the protein of interest/actin ratio. The significance of differences between means was determined using Student's *t*-test for unpaired samples. $P < 0.05$ was considered significant.

PTH injection

For PTH injection, four *Pdzk1*$^{+/+}$ and four *Pdzk1*$^{-/-}$ mice were fed a low-P_i diet for 4 days to increase the expression of the NaPi-IIa protein in the kidney. Mice were then injected i.p. with 0.5 μg/g BW 1–34 fragment of PTH (Sigma) or with 0.9% NaCl as control and sacrificed after 90 min. Kidneys were harvested rapidly and frozen until further use. Urine samples were collected before the injection and immediately before sacrifice. Blood samples were collected immediately before sacrifice. All urine and serum samples were frozen rapidly until further use.

Kidney slices

Kidney slice experiments were performed as described previously [2, 3]. Briefly, mice were kept on a low-P_i diet for 5 days prior to the experiments to increase NaPi-IIa expression in the kidneys. Mice were anaesthetized and perfused through the left ventricle with 50 ml warm (37 °C) sucrose/phosphate buffer to remove all blood from the kidneys. Kidneys were harvested rapidly, adhering connective tissue and extrarenal vessels removed and thin coronal slices (about 1 mm thick) cut. From each kidney six or seven slices could be prepared. Slices were transferred into 4 ml pre-warmed (37 °C) Hank's buffer (in mM: NaCl 110, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 1.8, Na-acetate 4, Na-citrate 1, glucose 6, L-alanine 6, NaH_2PO_4 1, Na_2HPO_4 3, $NaHCO_3$ 25, pH 7.4, gassed with 5% CO_2 and 95% O_2) and allowed to adapt for 10 min at 37 °C in a water bath before the start of the incubation. Slices were then left untreated (control) or incubated with 1–34-PTH (100 nM), 3–34-PTH (100 nM), 8-Br-cAMP (100 μM), 8-Br-cGMP (1 mM) or 1,2-dioctanoyl-sn-glycerol (DOG, 10 μM). During the whole course of the experiments all solutions were gassed with 5% CO_2/95% O_2 and the pH was kept constant at pH 7.4 ± 0.1.

Chemicals were from Sigma unless stated otherwise. All experiments were performed at least with two kidneys from two different animals. Untreated slices were used in all experiments as an internal control. For immunohistochemistry, kidney slices were transferred to a fixation solution (3% paraformaldehyde) at the end of the incubation [3] and fixed for 4 h on ice. After fixation, slices were rinsed few times with PBS, mounted on thin cork plates and frozen immediately in liquid propane cooled in liquid nitrogen.

Immunohistochemistry

Mice were fixed by perfusion through the left ventricle as described previously [7]. After perfusion, the kidneys were removed and cut into slices that were mounted on thin cork plates and frozen immediately in liquid propane cooled with liquid nitrogen. Cryosections (4 μm thick) mounted on chrome alum/gelatine-coated glass slides were processed for immunofluorescence. For NaPi-IIa, NHE3 and MAP17 immunofluorescence stains, sections were pretreated for 10 min with 3% defatted milk powder and 0.02% Triton X-100 (blocking solution) in PBS. After rinsing with PBS, sections were incubated with rabbit anti-rat NHE3 diluted 1:1,000, or with rabbit anti-rat serum against the NaPi-IIa protein diluted 1:500 or with rabbit anti-rat MAP17 diluted 1:1,000. For immunofluorescence detection of PDZK1 and D-AKAP2 sections were microwaved in a buffer containing 0.01 M citrate in distilled water at 30% power for 10 min. After rinsing with PBS and covering for 10 min with blocking solution, sections were incubated over night with anti PDZK1 antibody diluted 1:500 or with anti D-AKAP2 diluted 1:1,000. For antibodies against NHERF1/2, CFEX and the Na-sulphate transporter-1 (NaSi-1) sections were pretreated with 0.5% SDS in PBS for 7 min. After repeated rinsing with PBS they were covered for 10 min with blocking solution. Sections were then incubated overnight at 4 °C with anti NHERF antibody diluted 1:500, or with anti CFEX diluted 1:30 [13] or with rabbit anti-rat serum against the NaSi-1 protein [18] diluted 1:500. All primary antibodies were diluted in blocking solution and incubated overnight at 4 °C. Sections where then rinsed 3 times with PBS and covered for 45 min at room temperature in the dark with secondary antibody coupled to fluorescein isothiocyanate (FITC) or indocarbocyanine (CY3) (Dakopatts, Glostrup, Denmark). Double staining for NaPi-IIa and β-actin filaments was achieved by adding rhodamine-phalloidin (Molecular Probes, Eugene, Ore., USA, 1:50) in the solution containing the secondary antibodies. After rinsing with PBS, the sections were finally covered with glass-slips by using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo (2.2.2.)octane (DABCO, Sigma) as a fading retardant, and inspected using epifluorescence microscopy (Polyvar, Reichert-Jung).

Results

Steady-state expression of the NaPi-IIa cotransporter

Renal P_i reabsorption, localization and abundance of the NaPi-IIa protein are regulated tightly by dietary P_i intake. A diet rich in P_i reduces NaPi-IIa abundance, whereas a low-P_i diet increases NaPi-IIa expression [17]. We therefore first measured urinary P_i excretion and the

abundance and localization of NaPi-IIa in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice kept for 5 days (steady-state) on low- or high-P_i diets.

Pdzk1$^{+/+}$ and *Pdzk1*$^{-/-}$ mice fed a diet with a low (0.1%) inorganic P_i content for 5 days had a low urinary P_i excretion (expressed as mg P_i/mg creatinine), which was similar in both groups (Fig. 1a). The abundance of NaPi-IIa in the brush border membrane and its localization did not differ in kidneys from wild-type and PDZK1-deficient mice. Strong staining of the brush border membrane of early proximal tubules both of superficial and juxtamedullary nephrons was observed (Fig. 1b,c and Fig. 3).

A second group of mice received a diet with high (1.2%) inorganic P_i content for 5 days. In this group, total and fractional urinary P_i excretion was significantly higher in *Pdzk1*$^{-/-}$ mice than in wild-type animals (Fig. 2a, Table 1). Similarly, NaPi-IIa protein abundance in the brush border membrane was significantly lower in the *Pdzk1*$^{-/-}$ than in *Pdzk1*$^{+/+}$ mice

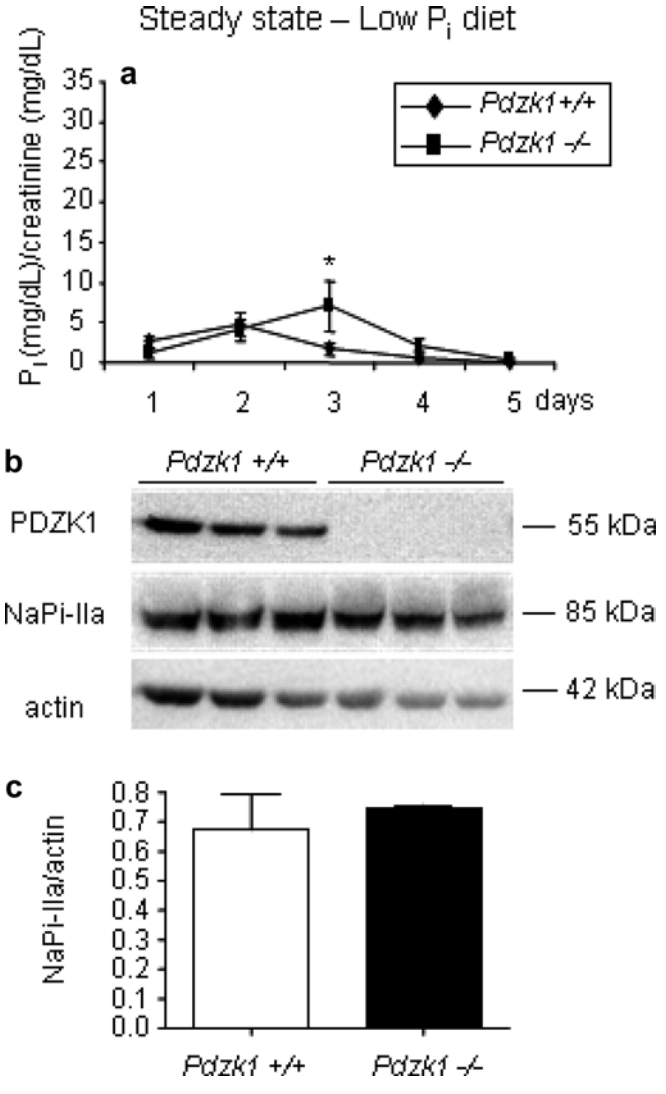

Fig. 1a–c Urinary P_i excretion and NaPi-IIa abundance in wild-type (*Pdzk1*$^{+/+}$) and PDZK1-deficient (*Pdzk1*$^{-/-}$) mice fed a low-P_i diet (0.1% P_i content) for 5 days. **a** Urinary P_i excretion relative to creatinine excretion (mg Pi/mg creatinine) measured in spontaneous urine samples collected daily at the same time from both genotypes. *Points* represent the average of the values obtained from three to five animals. *$P < 0.05$. **b**, **c** Western blot analysis for the Na/phosphate cotransporter (*NaPi-IIa*) in renal brush border membranes from wild-type and PDZK1 knock-out mice after 5 days on the low-P_i diet. *Upper panel*: PDZK1 protein expression; *middle panel*: NaPi-IIa expression. All membranes were stripped and reprobed for actin (*lower panel*) to normalize for loading. **c** Abundance of NaPi-IIa relative to that of actin

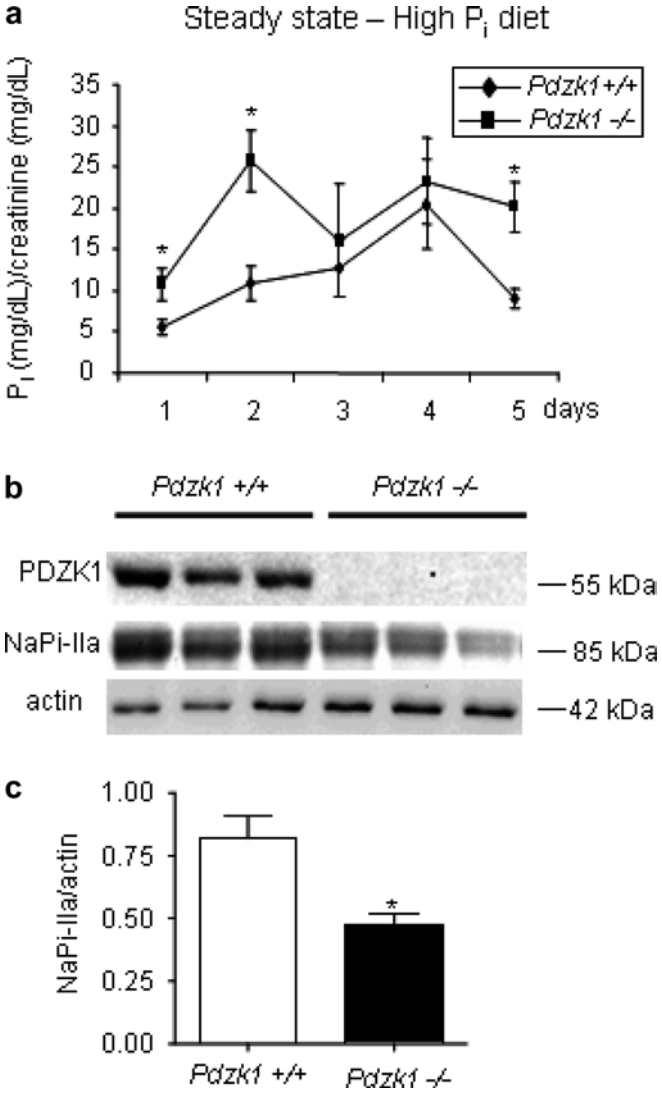

Fig. 2a–c Urinary P_i excretion and NaPi-IIa abundance in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice fed a high-P_i diet (1.2% P_i content) for 5 days. **a** Urinary P_i excretion relative to creatinine excretion (mg Pi/mg creatinine) measured in wild-type and PDZK1-deficient mice by collecting spontaneous urine samples. *Points* represents the average of the values obtained from three to five animals. *$P < 0.05$. **b**, **c** Western blot analysis for NaPi-IIa in renal brush border membranes from wild-type and PDZK1-knock-out mice after 5 days on the high-P_i diet. **b** Representative Western blot. *Upper panel*: PDZK1 expression; *middle panel*: NaPi-IIa expression. All membranes were stripped and reprobed for actin (lower panel) to normalize for loading. **c** Abundance of NaPi-IIa relative to that of actin. *$P < 0.05$

Table 1 P_i concentrations in serum and urine from wild-type (*Pdzk1*$^{+/+}$) and PDZK1-deficient (*Pdzk1*$^{-/-}$) mice under various dietary conditions. All values were obtained from animals after 5 days on either a low- or a high-$P_i$diet. Means ± SEM, $n = 6$ per group (FE_{Pi} fractional excretion of P_i)

	Pdzk1$^{+/+}$	*Pdzk1*$^{-/-}$	Significance
Serum (mg/dl)			
Low P_i	13.4 ± 0.2	14.0 ± 0.4	n.s.
High P_i	26.9 ± 3.0	24.6 ± 2.1	n.s.
Urine (mg/mg creatinine)			
Low P_i	0.08 ± 0.01	0.07 ± 0.01	n.s.
High P_i	9.00 ± 1.10	20.15 ± 3.10	$P = 0.019$
FE_{Pi} % (low P_i)	0.09 ± 0.02	0.08 ± 0.02	n.s.
FE_{Pi} % (high P_i)	6.02 ± 0.78	19.12 ± 3.56	$P = 0.020$

(Fig. 2b,c). The localization of NaPi-IIa in kidneys from wild-type and PDZK1-deficient mice displayed the same subcellular pattern and distribution along the nephron segments and generation (i.e. brush border membrane localization in the early proximal tubule of juxtamedullary nephrons) (Fig. 3). Thus, loss of PDZK1 seems to reduce urinary Pi-excretion and steady-state expression levels of NaPi-IIa in the brush border membrane of animals kept on a P_i-rich diet.

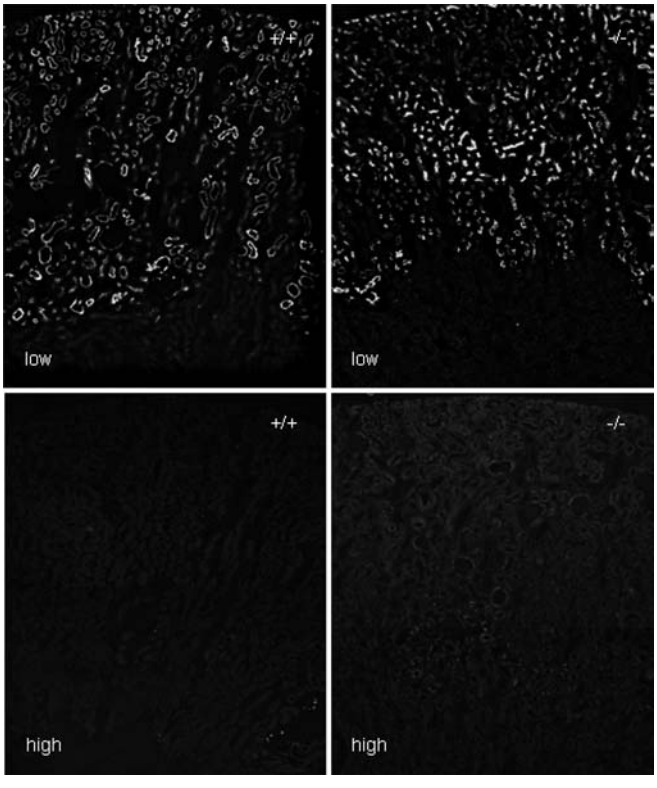

Fig. 3 Chronic adaptation to low- and high-P_i diets. Overview pictures from whole kidneys from wild-type and PDZK1-deficient mice kept for 5 days on the low- (0.1%) or high-Pi (1.2%) diet. No difference in the localization of the NaPi-IIa cotransporter could be detected. Original magnification 40×

Chronic adaptation to low- and high-P_i diet

As indicated from the steady-state results reported above, the chronic adaptation of renal P_i handling to changes in dietary P_i intake was similar in both groups of animals with the exception of lower NaPi-IIa protein levels during a P_i-rich diet. This was confirmed further by a direct comparison of the NaPi-IIa protein abundance in the brush border membrane of chronically (5 days) adapted animals. As summarized in Fig. 4, *Pdzk1* knock-out mice were as able as the wild-type animals to adapt to the different diets, with a stronger down-regulation of NaPi-IIa protein in the animals fed the high-P_i diet. Whether these lower levels of protein abundance reflect a reduction in NaPi-IIa trafficking or stability (i.e. increased turn-over in the membrane) remains unclear at this point.

Rapid adaptation to changes in dietary P_i intake

NaPi-IIa protein abundance and urinary P_i excretion adapt rapidly to acute changes in dietary P_i intake [17]. To establish whether PDZK1 plays a role in the rapid adaptive increase or decrease of NaPi-IIa abundance after ingestion of a diet either rich or low in P_i content, *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ were trained for 5 days to have access to their food for only 1 h in the morning. Some 4 h after the start of feeding, samples were collected for analysis (see Materials and methods). Animals from both genotypes were kept on low- or high-P_i diets and subdivided further into two groups, either continuing on a the prior low- or high-P_i diet or switched on day 5 to a high- or low-P_i diet respectively, thus resulting in four subgroups for each genotype.

The first group of mice, chronically adapted to the low-P_i diet, adapted rapidly to the high-P_i diet. Urinary P_i excretion was similar in both wild-type and knock-out groups on the low-P_i diet and after the switch to the high-P_i diet (Fig. 5a). These data were confirmed by Western blotting, showing similar protein abundance in the brush border membrane of both groups (data not shown). *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ control groups continued to receive a low-P_i diet and showed no difference in NaPi-IIa abundance and urinary P_i excretion as described above for steady-state conditions.

The second group of mice was switched from a chronic high- to the low-P_i diet. Again, urinary measurement of P_i excretion was comparable in wild-type and knock-out groups after the acute switch from the high- to the low-P_i diet (Fig. 5b). The higher P_i excretion under steady-state conditions in the knock-out mice group confirmed the data described above and reflected the lower protein abundance of the NaPi-IIa cotransporter in the brush border membrane as shown by Western blotting.

As shown in Fig. 6 the acute adaptation to the different diets resulted in a similar pattern of NaPi-IIa distribution in the kidney along the nephron. We found

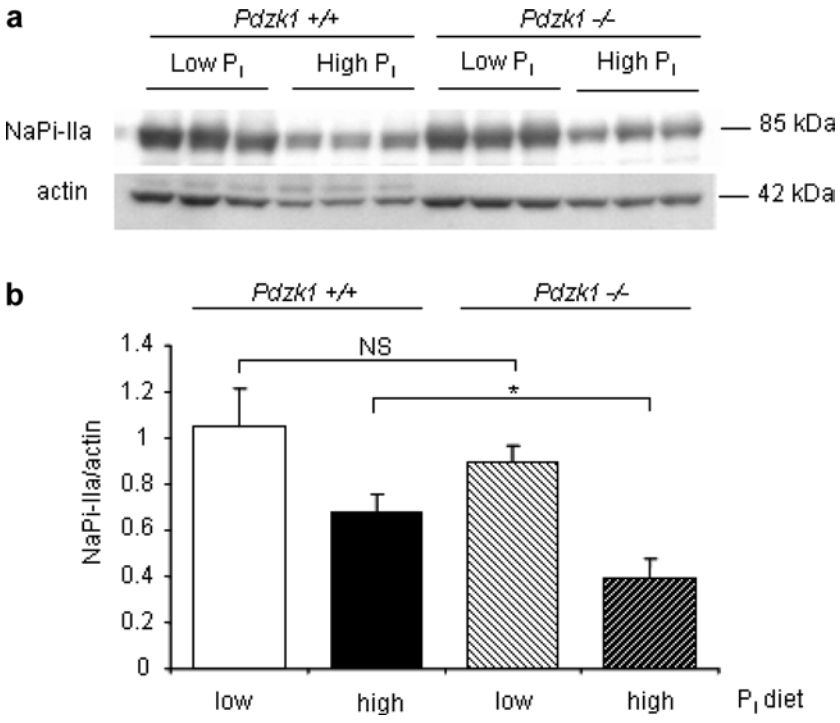

Fig. 4a,b Chronic adaptation of NaPi-IIa expression to low- and high-P_i diets. **a**, **b** Western blot analysis for NaPi-IIa in brush border membranes from kidneys of wild-type and PDZK1-knock-out mice chronically adapted to the low- and high-P_i diets. **a** Representative blot. All membranes were stripped and reprobed for actin to monitor loading. **b** Summary of NaPi-IIa abundance normalized to that of actin

no differences in the subcellular localization of the NaPi-IIa cotransporter between the wild-type and the knock-out animals in either the group of mice switched acutely from the low- to the high-P_i diet or in the group switched acutely from the high- to the low-P_i diet.

Rapid hormonal regulation of NaPi-IIa

PTH induces rapid down-regulation of NaPi-IIa by internalizing and subsequently degrading the protein [22]. PTH acts via receptors localized in the brush border membrane and on the basolateral side, activating several signalling cascades via protein kinases A and C [22]. cGMP-dependent internalization has also been described [1]. Thus, we tested whether the loss of PDZK1 affected PTH-induced internalization and degradation of NaPi-IIa in vivo and in freshly isolated kidney slices. *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice were given either 0.5 μg 1–34-PTH/g body weight i.p. or vehicle (0.9% NaCl). Mice had been adapted previously to the low-P_i diet to increase the renal expression of NaPi-IIa. 1–34-PTH reduced NaPi-IIa expression in the brush border membrane to a similar degree in both *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice (data not shown). To establish further whether the lack of the PDZK1 protein could affect the activation of these pathways or the subsequent internalization, further experiments were performed on freshly isolated kidney slices from *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice. The slices were incubated with 8-Br-cAMP (100 μM), 8-Br-cGMP (1 mM) or the protein kinase C activator DOG (10 μM) for 45 min. 1–34-PTH, which activates both cAMP/PKA and PLC/PKC dependent cascades, and fragment 3–34-PTH, which activates only the cAMP/PKA pathway, were also tested. All treatments resulted in the internalization of NaPi-IIa and a reduction of immunostaining due to the subsequent degradation. Figure 7a,c shows overviews of kidney sections from wild-type and knock-out animals with no detectable differences between the two groups. Also, as demonstrated at higher magnification (Fig. 7b,d), all treatments resulted in similar internalization of NaPi-IIa in both groups of animals.

Other proteins interacting with PDZK1

As shown previously, PDZK1 interacts via its four PDZ domains with various proteins expressed in the proximal tubule [10] such as NHE3, NHERF1, D-AKAP2, CFEX/PAT1 (SLC26A6) and the MAP17 protein. Western blot analysis detected no difference in the abundance of D-AKAP2 or MAP17 (data not shown). However, the abundance of CFEX/PAT1 (SLC26A6) was reduced in kidneys from *Pdzk1*$^{-/-}$ mice on the high-P_i diet (Fig. 8b,c). The abundance of NHERF1 in the brush border membrane of *Pdzk1*$^{-/-}$ was also increased, but only in animals on the high-P_i diet (Fig. 8b,c). The localization of all proteins investigated was undistinguishable in kidneys from wild-type and PDZK1-deficient mice (Fig. 8a).

Discussion

Renal P_i reabsorption depends critically on the expression and function of the Na^+/phosphate cotransporter NaPi-IIa as evident from studies in knock-out mice [20].

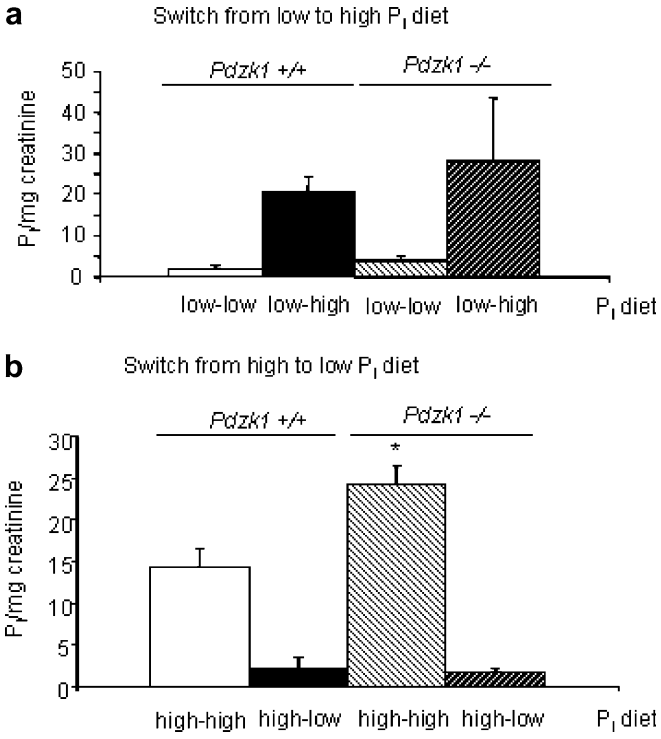

Fig. 5a,b Rapid adaptation of the NaPi-IIa cotransporter to acute changes in dietary P_i intake. Mice of both genotypes received a low-P_i (0.1%) diet (**a**) or a high-P_i (1.2%) diet (**b**) for 5 days. On day 5 some animals were then switched acutely to the other diet (high- or low-P_i diet, respectively), the remainder continued on the original diet. Urinary Pi excretion relative to creatinine excretion (mg Pi/mg creatinine) was measured in 12 wild-type and 12 PDZK1-deficient mice by collecting spontaneous urine samples. $*P < 0.05$ vs. other genotype

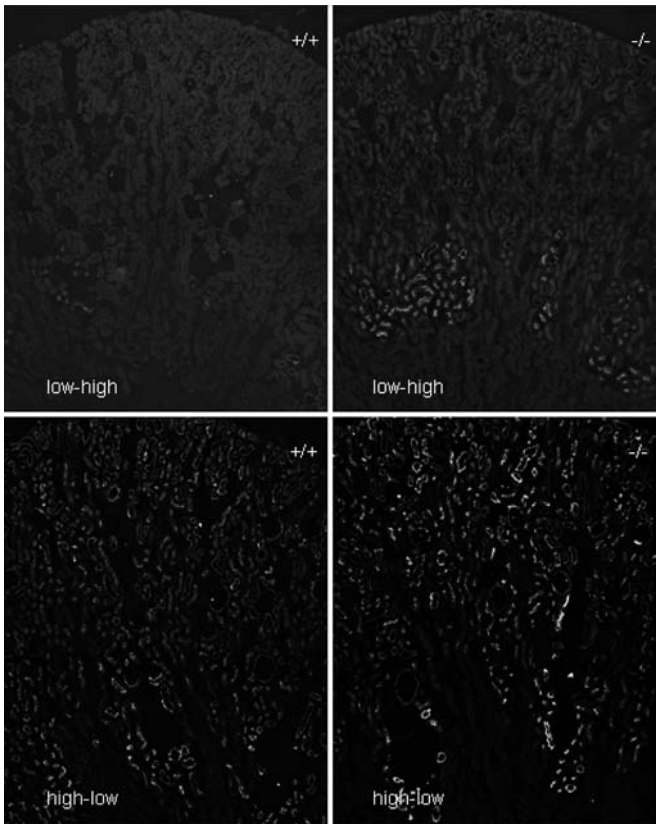

Fig. 6 NaPi-IIa specific immunoreactivity in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice kept for 5 days on either a low- (0.1%) or high-Pi (1.2%) diet and then switched acutely to the high- or low-P_i diet, respectively. Kidneys were harvested after 4 h and slices stained for NaPi-IIa-specific immunoreactivity

The activity of this transporter is regulated by a variety of hormones as well as dietary P_i intake [22]. An increase of activity is paralleled by new synthesis of transporter proteins and exocytotic insertion into the brush border membrane. In contrast, all stimuli identified so far leading to down-regulation induce retrieval of the transporter from the apical membrane, routing to lysosomes and subsequent degradation. Trafficking of newly synthesized protein to the apical membrane, anchoring in the brush border membrane and internalization and transport to the lysosomes require complex interactions with specialized proteins involved in the respective tasks. In an approach aimed at identifying such proteins important for the regulation of NaPi-IIa, several PDZ-domain-containing proteins have been found and shown to interact in vitro with NaPi-IIa through a classic PDZ-binding motif in the latter's C-terminus [8]. Among these proteins, NHERF1 and PDZK1 are of particular interest, as both proteins have been shown to interact with a variety of membrane proteins such as transporters or receptors and also with signalling proteins like protein kinase A-anchoring proteins or phospholipase C [10, 12, 19]. In addition, both proteins can also interact with each other [10, 26]. This has led to a concept suggesting that NHERF1 and PDZK1 may be part of an apical scaffolding protein network that may be important for positioning of several proteins in close proximity to each other. Such scaffolding may be important for processes like signalling or insertion into and retrieval from the apical membrane [9, 10].

The important role of NHERF1 in NaPi-IIa function and regulation has been highlighted by findings in OK cell cultures and in a NHERF1-deficient mouse model. However, little is known about the function of PDZK1 since no appropriate cell culture model has been available. The recent generation of a PDZK1-deficient mouse has now made it possible to investigate the role of PDZK1 in NaPi-IIa expression and regulation. The *Pdzk1*$^{-/-}$ mouse does not display an overt renal phenotype but does show a defect in liver lipoprotein metabolism due to a reduced expression of the high-density lipoprotein receptor scavenger receptor class B type I [15, 16].

In the present study we addressed four aspects of renal P_i handling in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice. (1) Urinary P_i excretion, expression and localization of NaPi-IIa under steady-state conditions. These experiments showed increased P_i loss in the urine under a high-P_i diet in PDZK1-deficient mice. (2). Acute and chronic adaptation of NaPi-IIa to changes in dietary P_i intake. Here we found

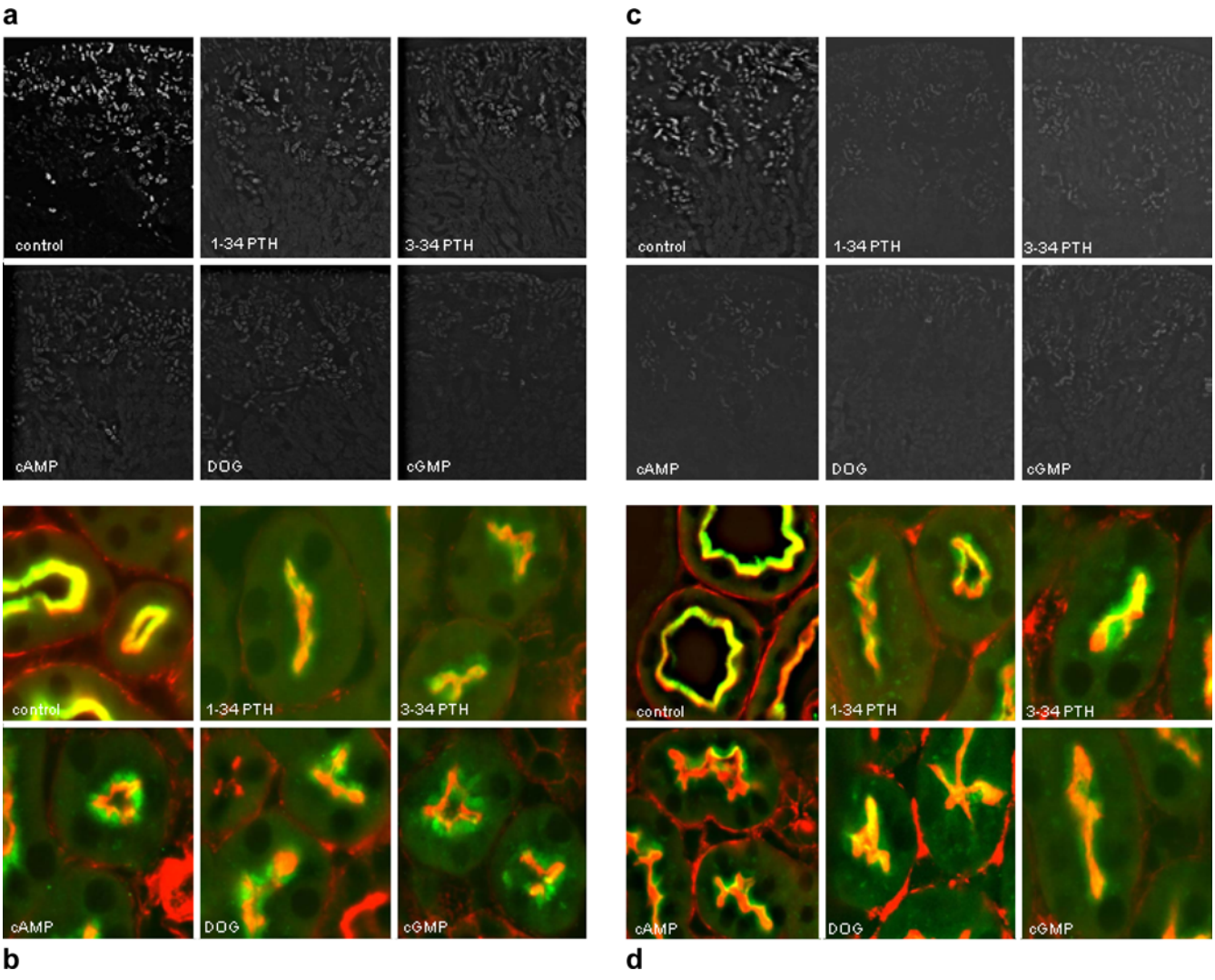

Fig. 7a–d Normal internalisation of NaPi-IIa in response to activation of various down-regulatory signalling cascades in freshly isolated kidney slices. Kidney slices from wild-type (**a**, **b**) and PDZK1-deficient mice (**c**, **d**) were prepared and incubated for 45 min either with control solution, parathyroid hormone (PTH) fragment 1–34, (*1–34-PTH*, 100 nM), 3–34-PTH (100 nM), 8-Br-cAMP (100 μM), the protein kinase C activator 1,2-dioctanoyl-sn-glycerol (*DOG*, 10 μM), or 8-Br-cGMP (1 mM). The sections were stained with an antibody against NaPi-IIa (*green*) and with rhodamine-phalloidin against *β*-actin filaments (*red*) as a marker for the brush border membrane. The high degree of overlap (*yellow*) between NaPi-IIa (green) and actin (red) under control conditions indicates localization of NaPi-IIa in the brush border membrane in both genotypes. After exposure to PTH fragments 1–34 and 3–34, cAMP, DOG or cGMP, NaPi-IIa cotransporter-related fluorescence in the wild-type brush border decreased, whereas the subapical cytoplasm showed a weak but distinct signal. There was no detectable difference between the wild-type and the knock-out animals. **a**, **c**: original magnification 40×, **b**, **d**: original magnification 800×

no difference between control and knock-out animals. (3). Acute down-regulation by internalization after activation of PTH receptors or separate activation of down-regulatory signalling cascades, again without any evident difference between control and knock-out mice. (4). The expression and localization of other PDZK1 interacting proteins than NaPi-IIa. These experiments showed the expression of the anion exchanger CFEX/PAT1 (SLC26A6) to be reduced and NHERF1/2 abundance increased under high-P_i diet conditions.

Taken together, these findings suggest that PDZK1 plays no role in the acute regulation of NaPi-IIa either by hormones or dietary changes in P_i intake. However, PDZK1 might be important for trafficking or stability of NaPi-IIa under conditions of a chronically high P_i intake. Interestingly, it has been shown that loss of NHERF1 reduces the adaptive insertion of NaPi-IIa into the membrane in response to a reduced dietary P_i intake [5, 28]. Thus, NHERF1 may be important for the trafficking or insertion of NaPi-IIa into the membrane whereas PDZK1 might serve a stabilizing function, which is unmasked only under conditions under which NaPi-IIa expression is low and not compensated by a high synthesis and insertion rate. The reduced abundance of the anion exchanger CFEX/PAT1 was only seen with the high-P_i diet. At the moment the physio-

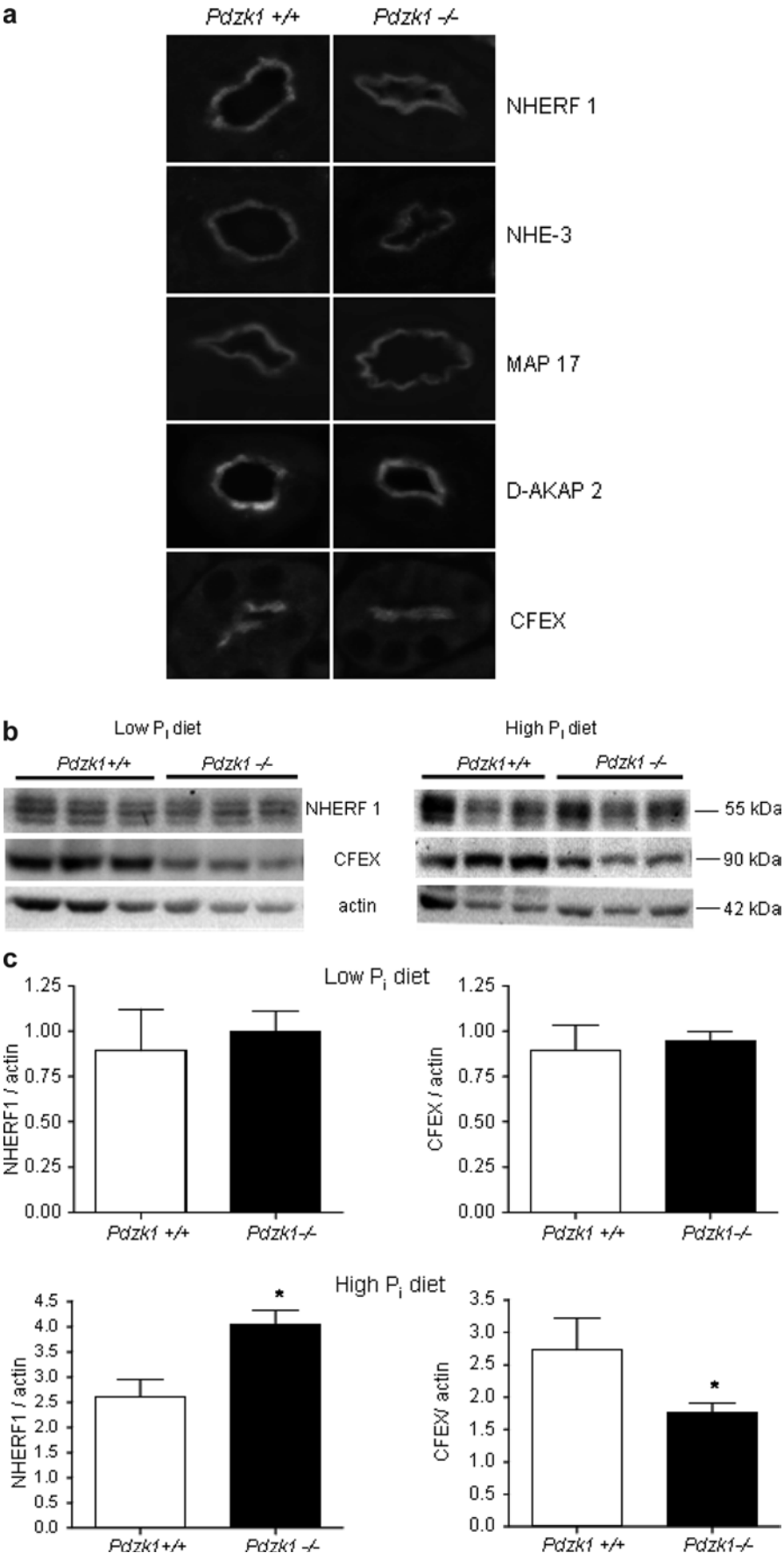

Fig. 8a–c Localization and abundance of other PDZK1-interacting proteins expressed in the proximal tubule. The localization and abundance of several proteins interacting in vitro with PDZK1 was examined in kidney sections (**a**) and in brush border membrane preparations (**b**). **a** Immunohistochemical staining for some of the NaPi-IIa interacting proteins. The localization of Na^+/H^+ exchanger-3 (*NHE3*), the NHE regulating factor-1 (*NHERF1*), the protein kinase A anchoring protein (*D-AKAP2*), the membrane-associated protein of 17 kDa (*MAP17*) and the chloride-formate exchanger (*CFEX*) was not altered in the knock-out mice, compared with wild-type. **b** Western blots of brush border membranes showing that the high-P_i diet enhanced the abundance of NHERF1 in the knock-out animals compared with wild-type and the decreased expression of CFEX. **c** Summary of NHERF1 and CFEX abundance normalized to that of actin in the brush border membrane of wild-type and PDZK1-deficient animals under low- and high-P_i diets. $^*P < 0.05$

logical significance of this finding is unclear as the physiological role of CFEX/PAT1 has not been clarified fully. The fact that NHERF1 abundance is increased under a high-P_i diet where NaPi-IIa abundance was also decreased suggests that NHERF1 up-regulation might be compensatory but not completely sufficient to prevent the reduction of NaPi-IIa expression.

Acute and steady-state levels of NaPi-IIa protein abundance are regulated by a variety of hormones including, most prominently, PTH and vitamin D_3. Even though we did not measure the concentrations of these hormones it appears very unlikely that loss of PDZK1 altered the levels of these hormones as detailed blood chemistry did not reveal any differences for other electrolytes influenced by these hormones e.g. Ca^{2+}, between *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice [15]. Thus, the observed changes in NaPi-IIa expression are most likely due to direct effects of PDZK1 deficiency in the proximal tubule.

In summary, loss of PDZK1 in the proximal tubule affects the expression of the major Na^+/phosphate cotransporter NaPi-IIa under conditions of a high P_i intake reducing its expression in the brush border membrane and increasing urinary P_i excretion. The acute and chronic adaptive and rapid hormonal regulation of NaPi-IIa was not altered in PDZK1-deficient mice. The subtle change in NaPi-IIa expression found in PDZK1-deficient mice may be due to compensatory or redundant processes as suggested by the fact that other PDZ proteins such as NHERF1 share many interacting partners with PDZK1. Combined ablation of both NHERF1 and PDZK1 may shed some light on the significance of their overlapping specificities.

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ERRATUM

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Expression and regulation of the renal Na/phosphate cotransporter NaPi-IIa in a mouse model deficient for the PDZ protein PDZK1

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Figure 8 reports results obtained with an antibody against the anion exchanger CFEX (SLC26A6). We became aware that this antibody cross-reacts with other proteins similar in size and localization to CFEX. Therefore we like to withdraw all data and conclusions related to the expression and localization of CFEX.

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7.2 Defective coupling of apical PTH receptors to phospholipase C prevents internalization of the Na+/phosphate cotransporter NaPi-IIa in NHERF1 deficient mice.

Paola Capuano, Desa Bacic, Marcel Roos, Serge M. Gisler, Gerti Stange, Jürg Biber, Brigitte Kaissling, Edward J. Weinman, Shirish Shenolikar, Carsten A. Wagner and Heini Murer

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Defective coupling of apical PTH - receptors to phospholipase C prevents internalization of the Na^+/phosphate cotransporter NaPi-IIa in NHERF1 deficient mice

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ABSTRACT

Phosphate reabsorption in the renal proximal tubule occurs mostly via the type-IIa sodium-phosphate cotransporter (NaPi-IIa) in the brush border membrane (BBM). The activity and localization of NaPi-IIa is regulated among other factors by parathyroid hormone (PTH). NaPi-IIa interacts in-vitro via its last three C-terminal amino acids with the PDZ-protein Na^+/H^+-Exchanger isoform 3 Regulating Factor 1 (NHERF1). Renal phosphate reabsorption in NHERF1 deficient mice is altered and NaPi-IIa expression in the BBM is reduced. In addition, it has been proposed that NHERF1 and NHERF2 are important for the coupling of PTH receptors (PTHR) to phospholipase C (PLC) and the activation of the protein kinase C (PKC) pathway.

Here we tested the role of NHERF1 in the regulation of NaPi-IIa by PTH in NHERF1 deficient mice. Immunohistochemistry and Western blotting demonstrated that stimulation of apical and basolateral receptors with PTH 1-34 led to internalization of NaPi-IIa in wildtype and NHERF1 deficient mice. Stimulation of only apical receptors with PTH 3-34 failed to induce internalization in NHERF1 deficient mice. Expression and localization of apical PTHR were similar in both wildtype and NHERF1 deficient mice. Activation of the protein kinase C and A dependent pathways with DOG or 8-Br-cAMP induced normal internalization of NaPi-IIa in wildtype as well as in NHERF1 deficient mice. The stimulation of PLC activity due to apical PTHR was impaired in NHERF1 deficient mice.

These data suggest that NHERF1 in the proximal tubule is important for PTH-induced internalization of NaPi-IIa and specifically couples the apical PTHR to PLC.

INTRODUCTION

In kidney, reabsorption of filtered inorganic phosphate occurs in proximal tubular cells, and the major player involved in this process is the sodium/phosphate cotransporter type IIa (NaPi-IIa), located in the brush border membrane (BBM) (23-25). The activity and abundance of this transporter is tightly regulated by different factors such as dietary phosphate intake, acid-base status and various hormones including steroid hormones, dopamine and parathyroid hormone (PTH) (23-25).

In the kidney, PTH interacts with a G-protein-coupled receptor (PTH1R) expressed both in the apical and basolateral membrane of the proximal tubule cells (21, 22, 32). Activation of apical or basolateral PTHR induces a strong and rapid down-regulation of NaPi-IIa due to the retrieval of the protein from the brush border membrane and its subsequent routing to the lysosomes for degradation (17, 26, 31). Furthermore, several PTH fragments have been identified that selectively activate apical or basolateral PTHR (21, 32). PTH 1-34 is active from both the apical and basolateral side, whereas PTH 3-34 is only effective on apically located receptors (22, 32). Apical PTHR predominantly couple to the phospholipase C (PLC) and protein kinase C (PKC) pathway, whereas basolateral PTHR activate the cAMP and PKA dependent pathway (22, 32). Both pathways ultimately lead to NaPi-IIa internalization and degradation (24).

The NaPi-IIa protein interacts via its last three C-terminal amino acid residues TRL with several PDZ-motif containing proteins, some of which co-localize in the brush border membrane together with NaPi-IIa (13, 14). These

proteins include among others the Na^+/H^+ regulatory factor 1 (NHERF1) and PDZK1 (6, 16).

NHERF1 was first identified as a regulatory factor of the sodium-proton-exchanger-3, and was later shown to be identical to the ezrin binding protein 50 (EBP50) (28, 36). A second isoform, NHERF2, has also been identified which is also localized in many epithelia but resides in a different compartment (33, 34). NHERF1 affects phosphate transporter activity and expression in the BBM in different experimental models. NaPi-IIa apical positioning is reduced *in vivo* in NHERF1 knock out mice (27). In addition, deletion of the C-terminal TRL motif in NaPi-IIa or overexpression of a soluble NHERF1 PDZ domain 1 (PDZ1) in the renal OK cell line disrupted apical NaPi-IIa localization (15)

NHERF1 contains two PDZ domains, PDZ1 and PDZ2) of which only PDZ1 is necessary for interaction with NaPi-IIa (19, 20). Moreover, NHERF1 forms part of a signalling complex in OK cells that contains the PTH1R, PLCβ and components of the actin cytoskeleton (19). It has been recently proposed that NHERF1 and NHERF2 are important for the coupling of PTH1R to phospholipase C (19, 20).

In order to test for the function of NHERF1 in the hormonal regulation of NaPi-IIa by PTH, one of its major physiological regulators, we examined the PTH-induced internalization and signalling pathway in NHERF1 deficient mice.

Our data show that the stimulation of PLC activity via apical PTH1R was impaired in NHERF1 deficient mice, suggesting that NHERF1 in the proximal tubule is important for proper PTH-induced internalization of NaPi-

IIa. Therefore, NHERF1 specifically couples the apical PTHR to PLC allowing activation of PLC dependent pathways and the subsequent regulation of major proximal tubular transport proteins.

EXPERIMENTAL PROCEDURES

Animal studies

Experiments were performed with ca. 24 weeks old and sex matched, wildtype mice (*Nherf1+/+*) and NHERF1 deficient mice (*Nherf1-/-*) of the same genetic background (C57BL6/J) weighing between 30 - 35 g. The generation and breeding of these mice have been described previously (27). For genotyping, tail DNA was prepared with the phenol:chloroform extraction method. For PCR, DNA samples were used at a concentration of 100 ng/µl. PCR was performed using Pfu DNA polymerase (Stratagene, USA). (Primer 1 -5'-CTCTGTTTATTCCCAGAAGGA-3'; Primer 2 -5'-CAAGAAGGCGATAGAAGGCGATG-3'; Primer 3 -5'-GAGCCAGGTTCTACCAGACGGATAAACTGG-3'). Amplicons generated by PCR were 1400 bp for the wild-type gene and 2400 bp for the knockout gene; heterozygous mice showed both amplicons.

Animals were housed in climatized animal facilities and received standard rodent diets (Kliba, NAFAG, Switzerland) with a high (1.2 %) or low (0.1 %) inorganic phosphate content and had free access to water. For some experiments mice were trained to receive food for only one hour a day to time food intake.

Spontaneous urine samples were collected daily at the same time and rapidly frozen until further analysis. Heparinized blood samples were collected from the vena cava immediately before sacrificing anesthetized animals. All samples were analyzed for inorganic phosphate and creatinine in urine (Jaffe method, Sigma) and blood (enzymatic test kit, WAKO, Germany) according to the protocol provided by the manufacturers.

All animal studies were performed according to Swiss Animal Welfare Laws and approved by the local Veterinary Authority (Kantonales Veterinäramt Zürich).

Kidney slices

Kidney slices experiments were performed as described previously (2-4). The viability of the slices for up to 1 hr has been previously demonstrated (4). Briefly, mice were anaesthetized with ketamine/xylazine i.p. and perfused through the left ventricle with 50 ml warm (37 °C) sucrose/ phosphate buffer (140 mM sucrose, 140 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) to remove all blood from the kidneys. Kidneys were rapidly harvested, adhering connective tissue and extra renal vessels were removed. Six to seven thin coronal slices (about 1 mm in thickness) were cut per kidney. Slices were transferred into 4 ml of pre-warmed (37 °C) Hank's buffer (110 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 4 mM Na-acetate, 1 mM Na-citrate, 6 mM glucose, 6 mM L-alanine, 1 mM NaH_2PO_4, 3 mM Na_2HPO_4, 25 mM $NaHCO_3$, pH 7.4, gassed with 5 % CO_2 and 95 % O_2) and allowed to adapt for 10 min at 37 °C in a water bath before the start of the incubation. Slices were then left untreated (control) or incubated with 1-34 PTH, 3-34 PTH, 8-Br-cAMP, or 1,2-dioctanoyl-sn-glycerol (DOG). During the whole course of the experiments all solutions were gassed with 5 % CO_2/ 95 % O_2 and the pH was kept constant at pH 7.4 ± 0.1. Kidney slices were further processed for immunohistochemistry or for brush border membrane preparations used for PLC activity assays and Western blotting (see below).

Immunohistochemistry

For immunohistochemistry, kidney slices were transferred for 4h on ice to a fixation solution (3% PFA) at the end of the incubation (2-5). After fixation, slices were rinsed three times with phosphate buffered saline (PBS), mounted onto thin cork plates and immediately frozen in liquid propane, cooled with liquid nitrogen. For NaPi-IIa immunostaining sections were pre-treated for 10 min with 3% defatted milk powder, 0.02% Triton X-100 in PBS ("blocking solution") to reduce non-specific binding of antibodies. Sections were then incubated with anti-rat NaPi-IIa rabbit antiserum (1:500). For PTH receptor staining, sections were pre-treated with 0.5% SDS in PBS for 5 min. After repeated rinsing with PBS, sections were incubated for 10 min with blocking solution. Sections were then incubated with an affinity-purified polyclonal antibody against PTH receptor (Covance Research Products, Richmond, CA, USA) at a dilution of 1:50. All primary antibodies were diluted in "blocking solution" and incubated overnight at 4°C. After overnight incubation, sections where rinsed three times with PBS and covered for 45 min at room temperature in the dark with swine anti-rabbit IgG conjugated to FITC (Dakopatts, Glostrup, Denmark) diluted 1:50 in PBS/milk powder, or goat anti-mouse IgG conjugated to CY3 (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) diluted 1:1000 in PBS/milk powder. Double staining of NaPi-IIa and β-actin filaments were achieved by adding rhodamine-phalloidin (Molecular Probes, Eugene, Ore., USA), respectively, at a dilution 1:50 in the solution containing secondary antibodies. Finally, the sections were rinsed three times with PBS, covered with a glass-slip using DAKO-Glicergel (Dakopatts) containing 2.5% 1,4-diazabicyclo [2.2.2.] octane (Sigma, St.

Louis, Mo., USA) as a fading retardant, and studied with an epifluorescence microscope.

Western blotting

Renal tissue for Western blotting was obtained either from incubated kidney slices or from kidneys prepared directly from mice. Mice were anaesthetized and perfused as described above. Kidneys were rapidly removed and frozen until further analysis. Frozen kidneys or kidney slices were used for brush border membranes preparation as described previously using the Mg^{2+}-precipitation technique (7). Brush border membrane protein concentration was measured (Biorad Protein kit) and 10 µg of protein were solubilized in Laemmli sample buffer containing 2 % v/v of 2-mercaptoethanol. Proteins were separated on 10 % SDS poly-acrylamide gels, and transferred to PVDF-membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 5 % milk powder in Tris-buffered saline plus 0.1% Tween20 for 60 min., the blots were incubated with the primary antibodies (rabbit anti-PDZK1 (14), 1:500, rabbit anti-NaPi-IIa, 1:6.000 (10), mouse monoclonal anti-actin (42 kDa, Sigma), rabbit anti-PTH receptor (Covance, Richmond, CA, USA), 1:1000, overnight at 4 °C or 2 h at room temperature. After washing and subsequent blocking, blots were incubated with the secondary IgG antibodies (donkey anti-rabbit 1:10.000 or sheep anti-mouse 1:10.000, respectively) conjugated with horseradish peroxidase (Amersham Life Sciences)) or with alkaline phosphatase (Promega) for 1 hr at room temperature. Antibody binding was detected with the peroxidase/luminal enhancer kit (Pierce, Rockford, USA) or with CDP-Star (Roche) respectively,

by means of the DIANA III - Chemiluminescence Detection System (Raytest, Germany). Images were analyzed with AIDA software (Advanced Image Data Analyzer (Raytest, Germany) to calculate the protein/actin ratio. All results were tested for significance using the unpaired student's t-test and only results with $P < 0.05$ were considered as statistically significant. All experiments were performed at least with six kidneys from three different animals. Untreated slices served in all experiments as an internal control.

P_i-uptake

The transport rate of phosphate [$^{32}P_i$] into brush border membrane vesicles was measured as described previously (7) at 25 °C in the presence of inward gradients of 100 mM NaCl or 100 mM KCl plus 0.1 mM K-phosphate. The phosphate uptake was determined after 90 s, representing initial linear conditions, and after 90 min to determine the equilibrium values.

PLC activity assay

Kidney slices from wildtype and *Nherf1-/-* mice were obtained as described above and left untreated (control) or incubated for 10 min. with 1-34 PTH or 3-34 PTH. Brush border membrane fractions were tested for PLC activity using the Amplex Red phosphatidylcholine-specific PLC assay kit (Molecular Probes, OR, USA) and using a dual scanning fluorescence microplate reader (GENios multifunctional reader, Tecan Trading AG, Switzerland). In this assay, PC-PLC activity is monitored indirectly through 10-acetyl-3,7-dihydrophenoxazine, a sensitive fluorogenic probe for H_2O_2. Initially, PC-PLC converts the phosphatidylcholine (lecithin) substrate to form phosphocholine

and diacylglycerol. After the action of alkaline phosphatase, choline hydrolyzed from phosphocholine is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2 in the presence of horseradish peroxidase reacts with Amplex Red reagent in a 1:1 stoichiometry to generate the highly fluorescent product resorufin. PLC activity was expressed in arbitrary florescence units per mg of protein.

RESULTS

In proximal tubular cells, PTH can interact either with an apical or basolateral G-protein coupled receptor, that triggers PLC and PKC or adenylyl cyclase and PKA, respectively (21, 22). Experimentally, it is possible to discriminate between these two pathways, since the fragment 1-34 of the hormone is activating both apical and basolateral receptors while the fragment 3-34 is only able to act via apical receptors (21, 22, 32). We employed this differential sensitivity to distinguish the effect of PTH on apical or basolateral receptors. We examined the downstream signalling pathways after the activation of the PTH1R in NHERF1 deficient mice in order to test for the function of NHERF1 in the regulation of NaPi-IIa.

Freshly isolated kidney slices were prepared from NHERF1 deficient and control mice and incubated *in vitro* with PTH 1-34 and PTH 3-34 (100 nM) for 1 h to test for PTH-induced internalization of NaPi-IIa by immunohistochemistry. This treatment has been previously shown to induce internalization of NaPi-IIa from the brush border membrane leading to transient accumulation in the subapical compartment and subsequent routing to lysosomes for degradation (1, 3, 4, 11). As shown in **Fig. 1A**, incubation of kidney slices with PTH 1-34 allowed a normal internalization of NaPi-IIa in kidneys from NHERF1 deficient and control mice. Higher magnification images showed clearly the sub-apical appearance of NaPi-IIa after treatment with the hormone indicating that the application of PTH, in both wildtype and NHERF deficient mice resulted in the retrieval of the sodium phosphate cotransporter. In contrast, activation of only apical PTH receptors with 3-34

PTH failed to induce a visible internalization of NaPi-IIa in slices prepared from NHERF1 deficient mice but caused retrieval in kidney obtained from wildtype mice (**Fig. 1B**).

Brush border membranes (BBM) were prepared from kidney slices treated with 1-34 PTH or 3-34 PTH or left untreated and subjected to immunoblotting. NaPi-IIa protein abundance was lower in control slices prepared from NHERF1 deficient mice than from wildtype mice as previously shown (27) (**Fig. 2**). Both PTH fragments significantly reduced NaPi-IIa protein in the BBMV from wildytpe mice. In NHERF1 deficient mice PTH 1-34 displayed a non-significant decrease in the BBM from, whereas PTH 3-34 was without effect (**Fig. 2**) consistent with the observations from immunohistochemistry (**Fig. 1**).

Apical PTH receptors and pharmacological activation of PKC with the diacylglycerol analog 1,2-dioctanoyl-sn-glycerol (DOG) induce NaPi-IIa internalization from the BBMV through an PLC and PKC dependent pathway (4, 32). Therefore we tested if activation of PKC with DOG induced NaPi-IIa retrieval in freshly isolated kidney slices. Slices were also incubated with 8-Br-cAMP to activate the PKA dependent internalization of NaPi-IIa as a positive control. Activation of PKA and PKC by 8-Br-cAMP or DOG, respectively, induced internalization of NaPi-IIa in slices from NHERF1 wildtype mice, as well as from NHERF1 deficient animals (**Fig. 3**). Thus, the failure of PTH 3-34 to retrieve NaPi-IIa is not based on a loss of the endocytic machinery to respond to a PKC-dependent stimulus. The defect must rather rely on the

activation of the apical PTH receptor itself or the transduction of the signal from the receptor to PKC.

Next, we investigated the expression levels and localization of the PTH receptor on the apical side of the proximal tubular cells by means of immunoblots from brush border membranes and immunohistochemistry on kidney slices form non treated animals. When compared to wildtype mice both the expression level and the localization of the PTH receptor were preserved in NHERF1 knock-out mice (**Fig. 4**). As NHERF1 and NHERF2 both anchor PTH receptors to PLC in membrane domains (19, 20), phospholipase C activity was measured in kidney slices from NHERF1 deficient mice to test if signal transduction of activated PTH receptors is depending on interaction with NHERF1. Therefore, kidney slices were incubated for 10 min with 1-34 or 3-34 PTH. Thereafter, BBMV were prepared and PLC activity measured. The activity of PLC clearly increased in 1-34 and 3-34 PTH treated samples from wildtype mice as expected. On the contrary, no rise in PLC activity was found in kidney slices from knock-out mice and total PLC activity rather decreased significantly (**Fig. 5**). This strongly suggests a defective coupling of the apical PTHR to PLC in NHERF1 deficient mice and a requirement of NHERF1 for PLC activation in response to stimulation of apical PTH receptors.

NaPi-IIa is also rapidly down-regulated and internalized after the intake of a phosphate rich diet (8, 18), a process that is independent from PTH (30). To investigate if NHERF1 is involved in this rapid down-regulatory adaption, NaPi-IIa localization, activity, and abundance were assessed 4 h after giving a phosphate rich diet. Mice were trained to engulf food during one hour in the morning. They were kept for 5 days on a low P_i diet before switching the diet

to a high phosphate diet on the 6th day. Some mice were continued on the low P_i diet and served as controls. As shown in **Fig. 6**, the dietary regulation of the cotransporter was not altered in NHERF1 deficient mice compared to control mice: i) immunohistochemistry showed a comparable localization of the NaPi-IIa protein in sections of kidney from mice chronically adapted to a low phosphate diet and acutely adapted to a high phosphate diet; ii) similarly, immunoblotting of brush border membrane fractions obtained from the same groups of animals revealed no difference in the adaption to the dietary change; iii) Na^+-dependent P_i uptake activity into brush border membrane vesicles from the same groups of mice was identical in both groups of mice. A similar reduction in the transport activity was detected in wildtype and NHERF1 knock out mice that were acutely adapted to a high phosphate diet compared to samples from mice chronically adapted to the low phosphate diet (**Fig. 6**).

DISCUSSION

The sodium phosphate cotransporter type IIa (NaPi-IIa), located in the brush border membrane of the proximal tubular cells, represents the major renal phosphate absorptive mechanism (24, 25). Its activity is tightly regulated by dietary intake of phosphate, acid-base status, and several hormones including PTH (24, 25). In order to identify interacting proteins that may be involved in regulation, membrane targeting and retraction of NaPi-IIa, we applied previously a yeast-two-hybrid screen with the cytosolic C-terminus of mouse NaPi-IIa that contains the canonical TRL PDZ-domain binding consensus sequence (14). Among others, the PDZ proteins NHERF1 and PDZK1 were identified. These two proteins highly interact with NaPi-IIa in several in-vitro assays and importantly colocalized with NaPi-IIa in the proximal tubular brush border membrane (12-14). Using a PDZK1 deficient mouse model, we have shown that PDZK1 is important for the expression of NaPi-IIa under conditions of high phosphate intake probably by stabilizing the protein in the membrane (8). NHERF1, in contrast, appears to be important for membrane insertion or trafficking of NaPi-IIa into the membrane. Deleting the very last TRL from the C-terminus of NaPi-IIa or overexpressing the interacting PDZ1 domain of NHERF1 impairs apical expression of NaPi-IIa in the OK cell model (15). In the NHERF1 deficient mouse, NaPi-IIa expression in the brush border membrane is reduced and mice present with a mild hyperphosphaturia (27). Adaption to a low phosphate diet involves insertion of NaPi-IIa protein into the brush border membrane. This process is defective in

NHERF1 deficient mice (35) and in a primary proximal tubular cell line derived from NHERF1 null mice (9).

Based on transfected cell line models, this scaffolding protein may be important for the formation of a multi-protein complex allowing the coupling of the PTH receptor to its downstream effector, phospholipase Cβ (PLCβ) (19, 20). As NaPi-IIa binds NHERF1 and is internalized by a PLC and PKC dependent pathway, we were interested in investigating the role of NHERF1 in the regulation of the sodium phosphate cotransporter by the parathyroid hormone. Taking advantage of the availability of a mouse model deficient for NHERF1, *in vitro* experiments indicate, that NHERF1 in the proximal tubule is important for a proper PTH induced internalization of the NaPi-IIa cotransporter from the brush border membrane. However, ablation of NHERF1 does not generally impair internalization as evident from three sets of experiments: i) internalization of NaPi-IIa occurs with PTH 1-34 which is also acting on basolateral PTH receptors, ii) internalization can be induced with pharmacological activators of the PKA and PKC pathways, and iii) internalization and downregulation of NaPi-IIa was normal after an acute switch to a high phosphate diet. Thus, the impairment is specific for the activation of apical PTH receptors in contrast to recent results obtained with mouse models deficient for endocytic receptor protein megalin or for its chaperone RAP where internalization in general was attenuated (1, 3).

Our experiments demonstrate that the failure to internalize NaPi-IIa in response to PTH 3-34 is caused by the defective coupling of the apical PTH receptor to phospholipase C. Expression and localization of apical PTH receptors were not affected by the loss of NHERF1, but their ability to

increase PLC activity upon stimulation. PLC activity was even slightly but significantly reduced which could be due to a cAMP-mediated inhibition by a negative feedback mechanism on PLC activity (29). Stimulation of protein kinase C, one of the down-stream effectors of PLC, could still induce NaPi-IIa internalization. Thus, NHERF1 is most likely required for the coupling of PLC to apical PTH receptors in the proximal tubule. In contrast, basolateral PTH receptors stimulate adenylate cyclase activity and induce NaPi-IIa internalization via a cAMP/ PKA dependent pathway (22, 32). This alternative coupling allows basolateral PTH receptors to regulate NaPi-IIa even in the absence of a functional apical PTH receptor-NHERF1-PLC-NaPi-IIa complex.

In proximal tubule cell models NHERF1 assembles a PDZ-based multiprotein signalling complex including Ezrin, NHE3, and PKA, which facilitates the phosphorylation of NHE3 by PKA, thereby inhibiting the activity of this transporter (6, 16, 28, 36). In contrast, our data obtained from experiments on freshly isolated kidney slices *in vitro* and from whole animals *in vivo* suggest that the PKA/Ezrin/NHERF1 complex is not essential for the regulation of NaPi-IIa by PKA, as a normal PKA mediated internalization of NaPi-IIa and residual phosphaturia was observed. The compensatory upregulation of other proteins involved in the PKA-dependent regulation can not be completely ruled out, but defective PKA-dependent regulation of NHE3 activity has been demonstrated in NHERF1 deficient mice thereby pointing to the requirement of NHERF1 in this pathway (37). The normal internalization of NaPi-IIa following the PKA-dependent pathway most likely explains the partially preserved phosphaturic effect of PTH 1-34 in NHERF1 deficient mice.

However, due to the loss of the PKC-dependent pathway PTH can not exert its full phosphaturic effect.

In summary, loss of NHERF1 affects the PTH-induced internalization of the major renal Na^+/phosphate cotransporter NaPi-IIa *in vitro*. The disturbance is most likely caused by the defective coupling between the apical PTH receptor and PLC. Ablation of NHERF1 fails to bring PTH receptors in close proximity to PLC and hence fails to activate the subsequent PKC-dependent cascade which leads to NaPi-IIa internalization and its degradation. This is the first *ex vivo in vitro* evidence that NHERF1 affects the function of a G-protein coupled receptor underlining the importance of scaffolding proteins for the organisation of polarized signalling in epithelia.

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FIGURE LEGENDS

Figure 1. Internalization of NaPi-IIa from the brush border membrane in response to PTH.

Kidney slices from wildtype and *Nherf1-/-* were prepared and incubated for 45 min. either with control solution or with parathyroid hormone fragments 1-34 (**A**) or 3-34 (**B**), both at a concentration of 100 nM. The sections were stained with an antibody directed against NaPi-IIa. After exposure to PTH 1-34, NaPi-IIa related fluorescence was similarly decreased in slices from both wildtype and *Nherf1-/-* mice with a concomitant increase of the signal in the subapical compartment (lower panel). After exposure to PTH 3-34, NaPi-IIa related fluorescence was decreased in slices from wildtype mice, but not in *Nherf1-/-* mice when compared to the control and to the wild type **(B)**. Original magnifications 40x (upper panel) 800x (lower panel)

Figure 2. Retrieval of NaPi-IIa protein from the brush border membrane after treatment with PTH 1-34 and PTH 3-34.

Brush border membrane fractions were isolated from kidney slices treated with 1-34 PTH, 3-34 PTH or left untreated as control. NaPi-IIa and actin abundance were determined by immunoblotting. The ratio between NaPi-IIa and actin is expressed as percentage of the control. Levels of significance were: n.s. not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.001$.

Figure 3. Intact PKA and PKC mediated NaPi-IIa internalization.

Kidney slices from wildtype and *Nherf1-/-* mice were prepared and incubated for 45 min. either with control solution, the PKA activator 8-Br-cAMP (100 µM) or the PKC activator 1,2-dioctanoyl-sn-glycerol, DOG (10 µM). The sections were stained with an antibody against NaPi-IIa (green) and with rhodamine-phalloidin against β actin filaments (red) as a marker for the brush border membrane. The high degree of overlap (yellow) between NaPi-IIa (green) and actin (red) under control conditions indicates apical localization of NaPi-IIa in both genotypes. After exposure to 8-Br-cAMP or DOG NaPi-IIa cotransporter related fluorescence in the wildtype brush border shifted towards the subapical compartment. There was no detectable difference between the wildtype and the knock-out animals. Original magnification 800x.

Figure 4. Preserved apical PTH receptor expression in *Nherf1* -/- proximal tubules

(A) Western blot against the PTH1R and actin demonstrated that the abundance in the brush border membrane is unchanged in NHERF1 -/- mice compared to wildtype. Membranes were stripped and reprobed for actin as a loading control. **(B)** Summary of PTH1R and actin ratios. No significant difference was found. **(C)** Immunohistochemical staining of the PTH1R in the proximal tubule. No obvious difference in the localization of the PTH1R in the brush border membrane in NHERF1 -/- and wildytpe mice was observed. Original magnification 800x.

Figure 5. Loss of PLC activation in response to PTH in NHERF1 deficient mice

Phospholipase C activity was measured in brush border membranes obtained from kidney slices samples incubated with either a control solution, PTH1-34 (100 nM) or PTH 3-34 (100 nM) for 10 min.. PLC activity increased in PTH treated slices from wildtype mice whereas it even decreased in slices from NHERF1 deficient mice. The figure shows the summary of five different experiments. PLC activities are depicted in arbitrary units and were normalized non-stimulated control slices. Levels of significance were: n.s. not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.001$.

Figure 6. Normal acute down-regulation of NaPi-IIa in response to high dietary phosphate intake

Mice were kept for 5 days on a low Pi diet (0.1 %) and then switched acutely to a high Pi diet (1.2 %). Kidneys were harvested after 4 h. **(A)** NaPi-IIa specific immunoreactivity was observed in kidneys from *Nherf1*+/+ and *Nherf1-/-* and no difference detected. **(B,C)** BBMV were prepared and tested for NaPi-IIa and actin. The NaPi-IIa/ actin ratio was calculated and is shown as summary bar graphs. The results demonstrate that NaPi-IIa expression is adapted to a similar extent in both groups. **(D)** Na^+-dependent P_i-uptake was measured in BBMV prepared from the same group of mice. Switching to a high P_i diet decreased P_i uptake in both groups similarly and no difference was detected in the adaption between both groups. Levels of significance were: n.s. not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.001$.

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Fig. 1A

Capuano et al.

Nherf1 +/+

Control | PTH 1-34 | PTH 3-34

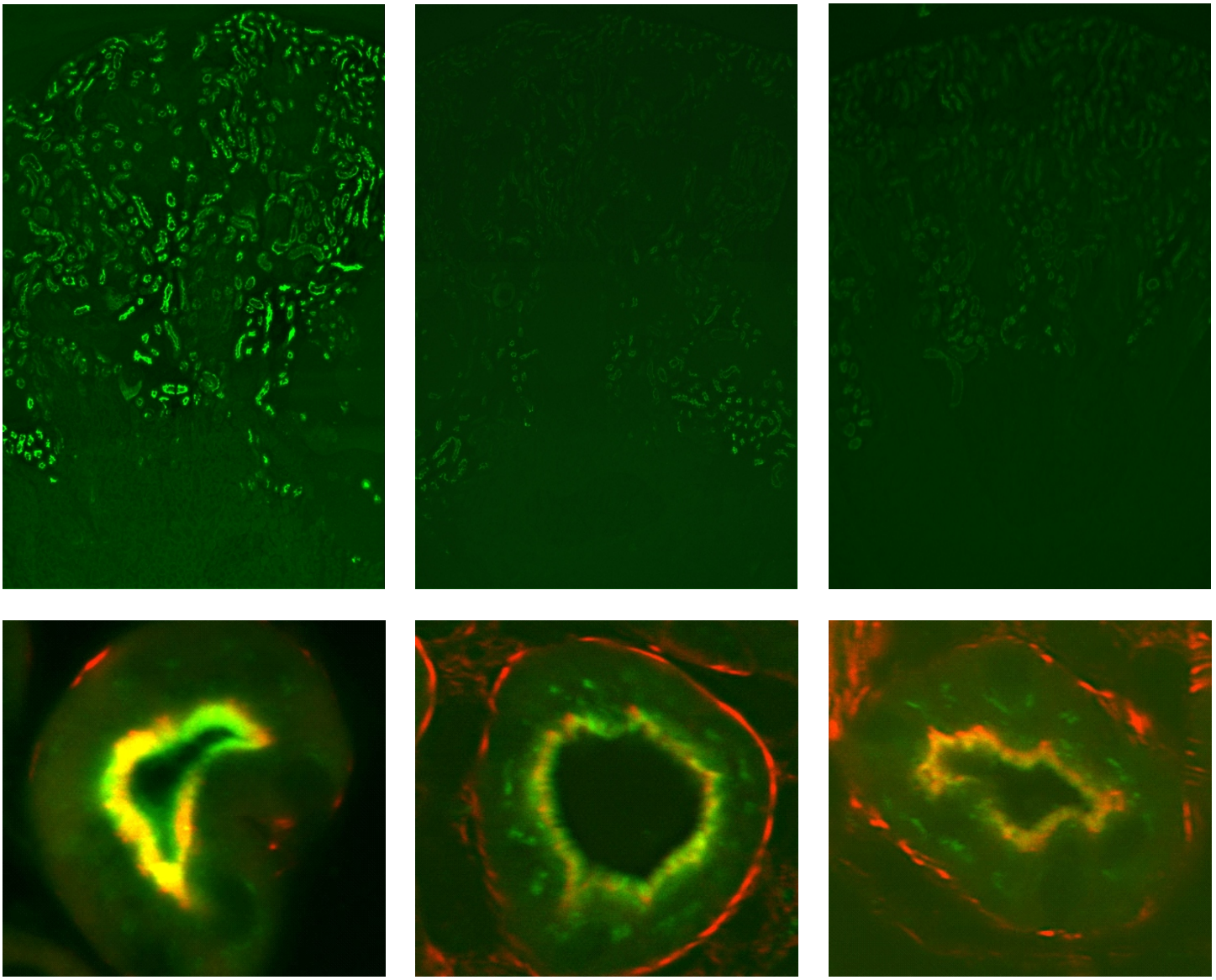
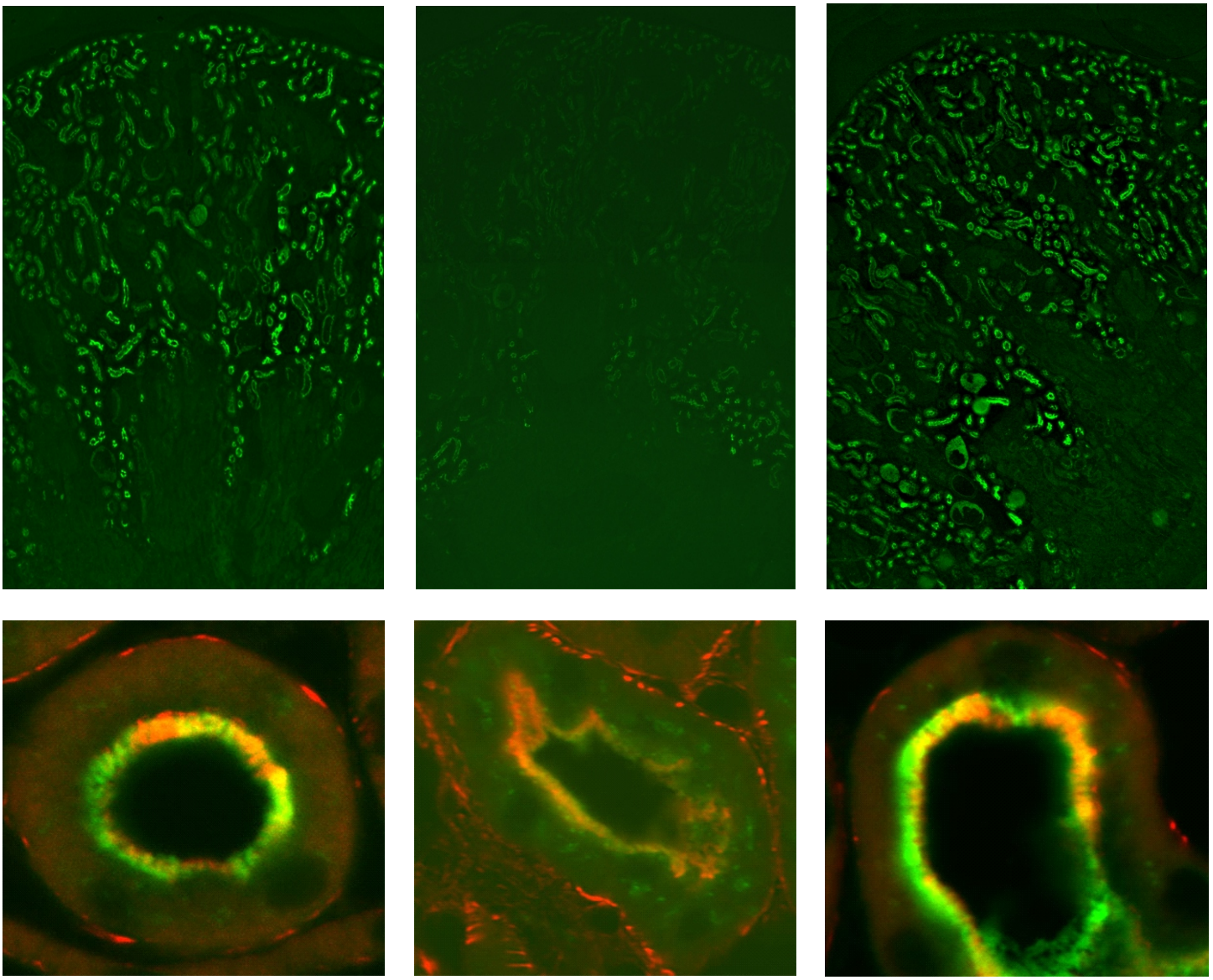


Fig. 1B

Capuano et al.

Nherf1 -/-

Control | PTH 1-34 | PTH 3-34



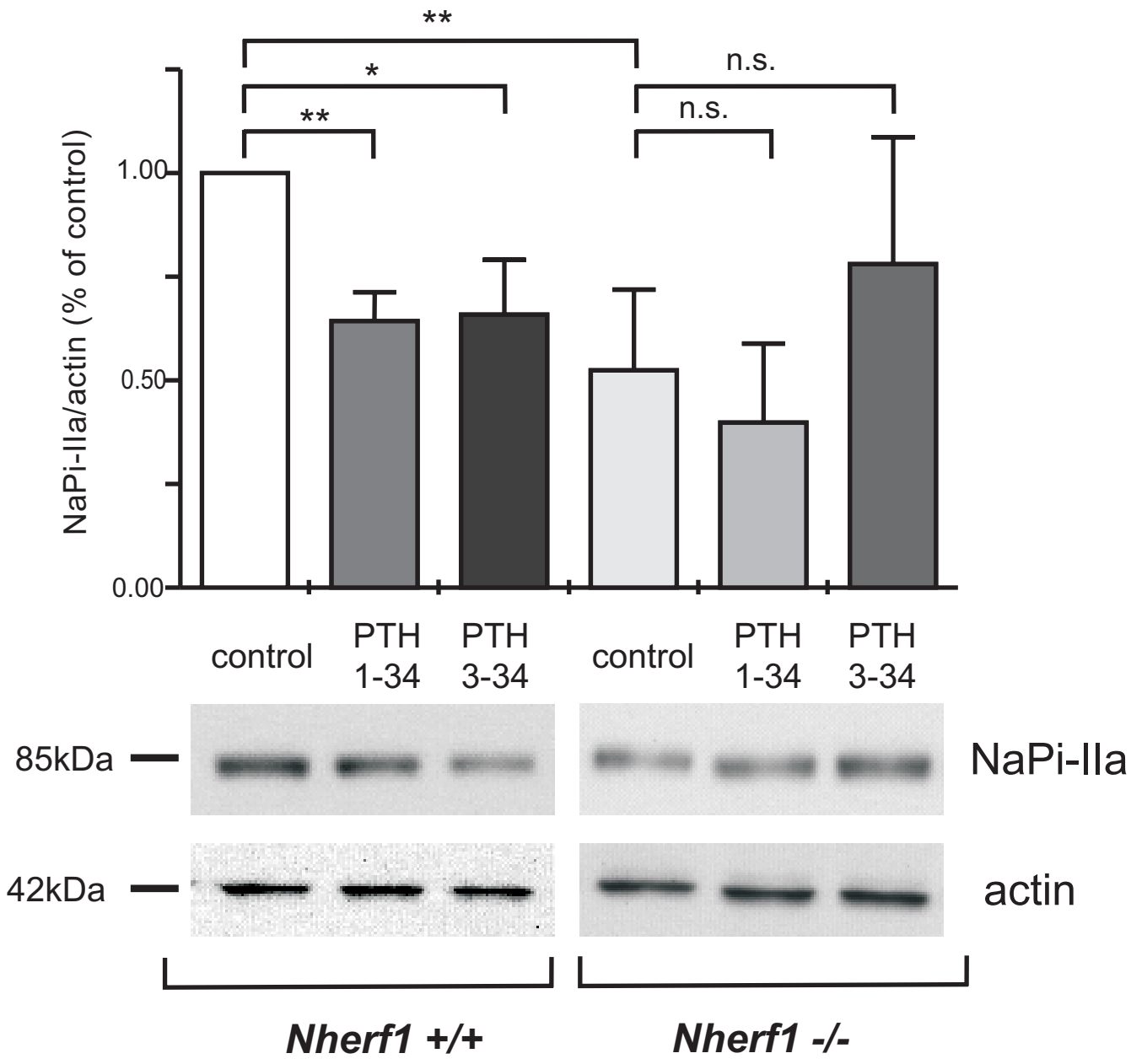

**
*
**
n.s.
n.s.
NaPi-IIa/actin (% of control)
1.00
0.50
0.00
control
PTH 1-34
PTH 3-34
control
PTH 1-34
PTH 3-34
85kDa
NaPi-IIa
42kDa
actin
Nherf1 +/+
Nherf1 -/-

Fig. 3

Capuano et al.

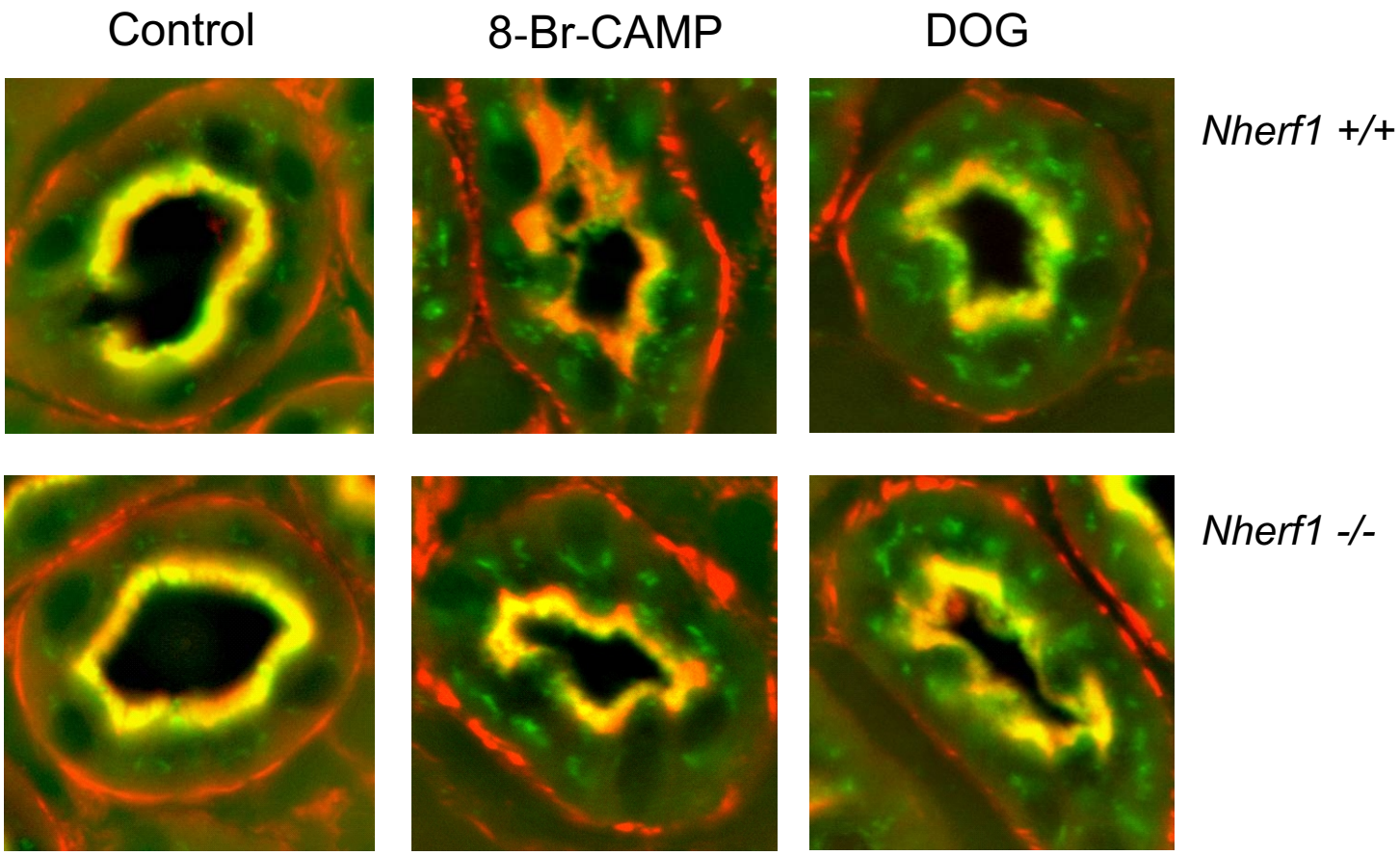
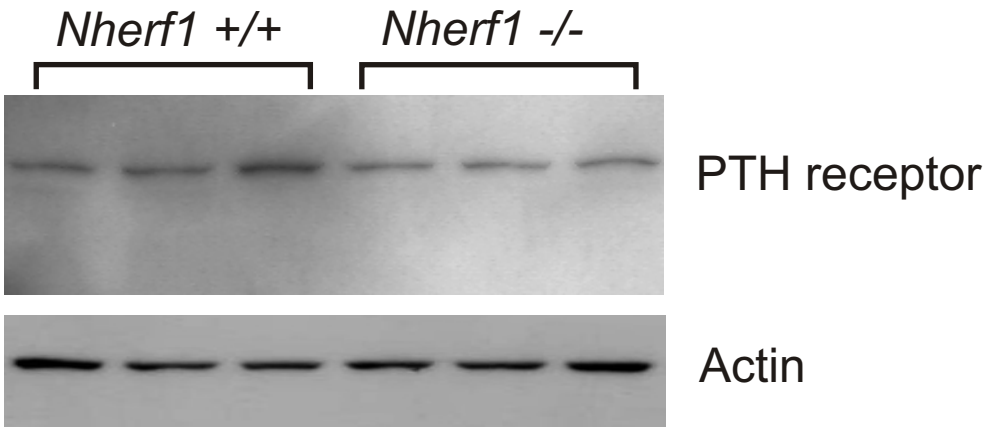


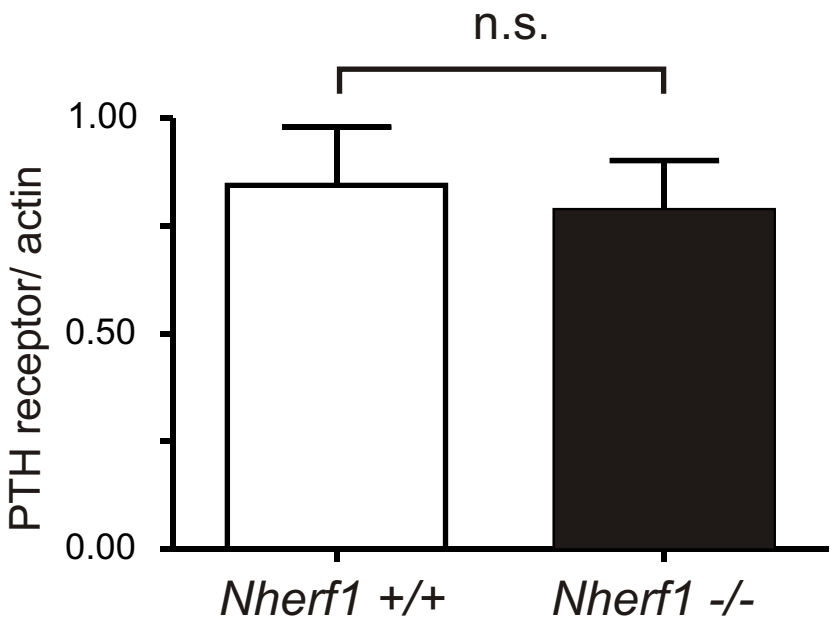
Fig. 4

Capuano et al.

A.



B.



C.

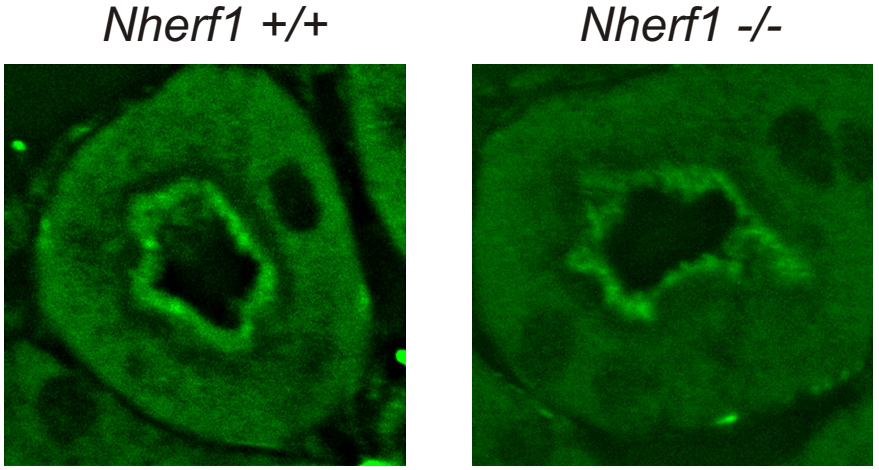


Fig. 5

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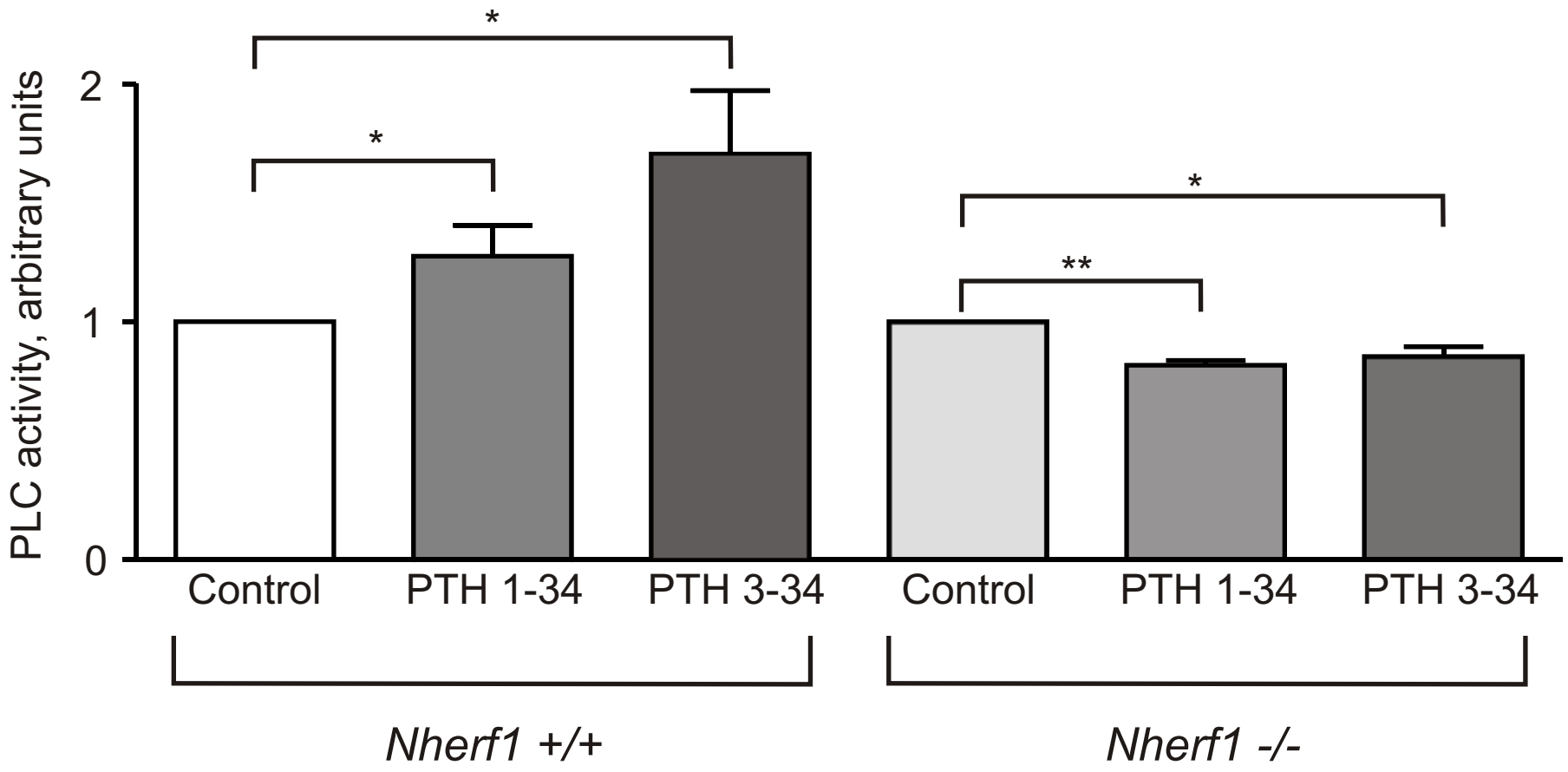
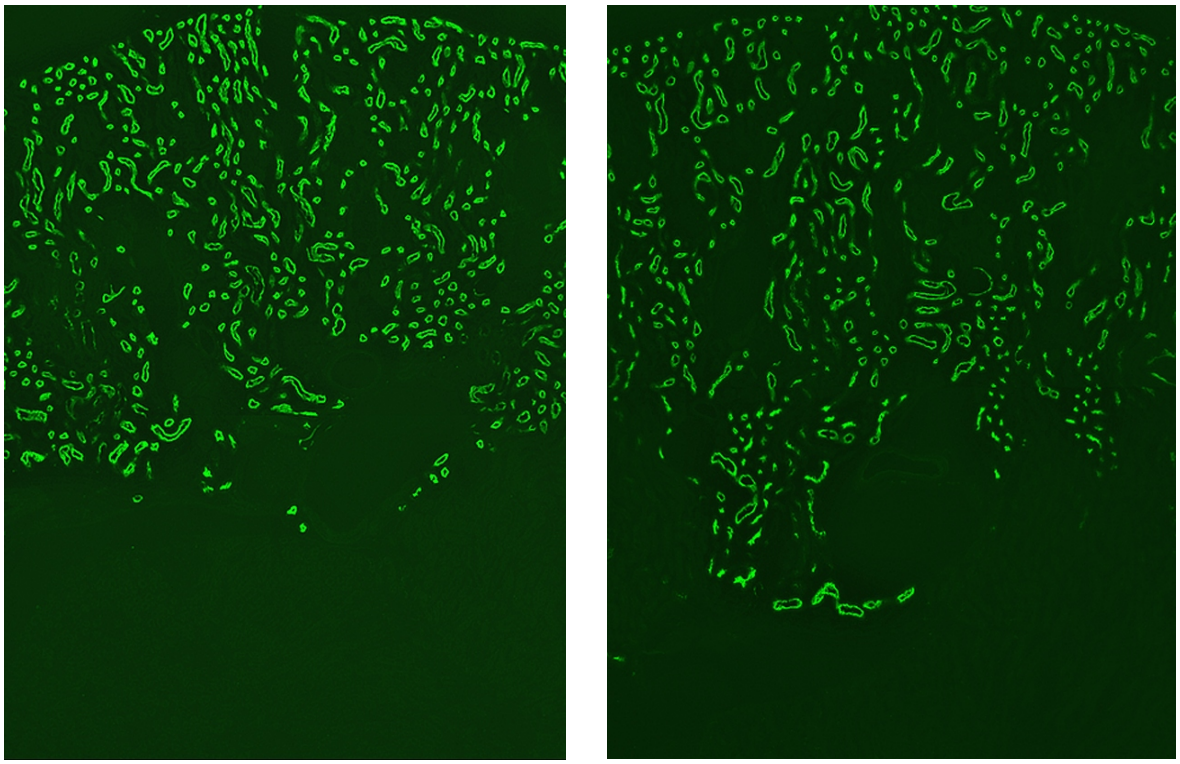


Fig 6A

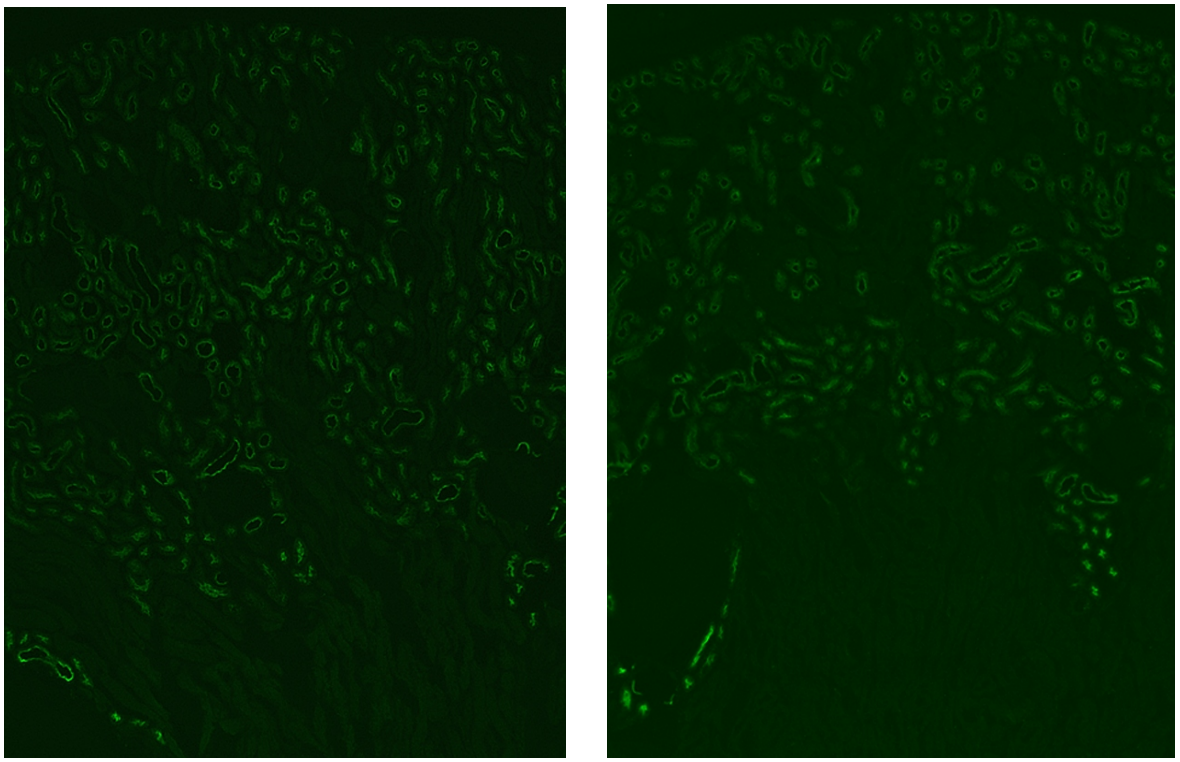
Capuano et al.

Nherf1 +/+

Nherf1 -/-



5d
low P_i diet



5d
low P_i diet
+
4h
high P_i diet

Fig. 6

Capuano et al.

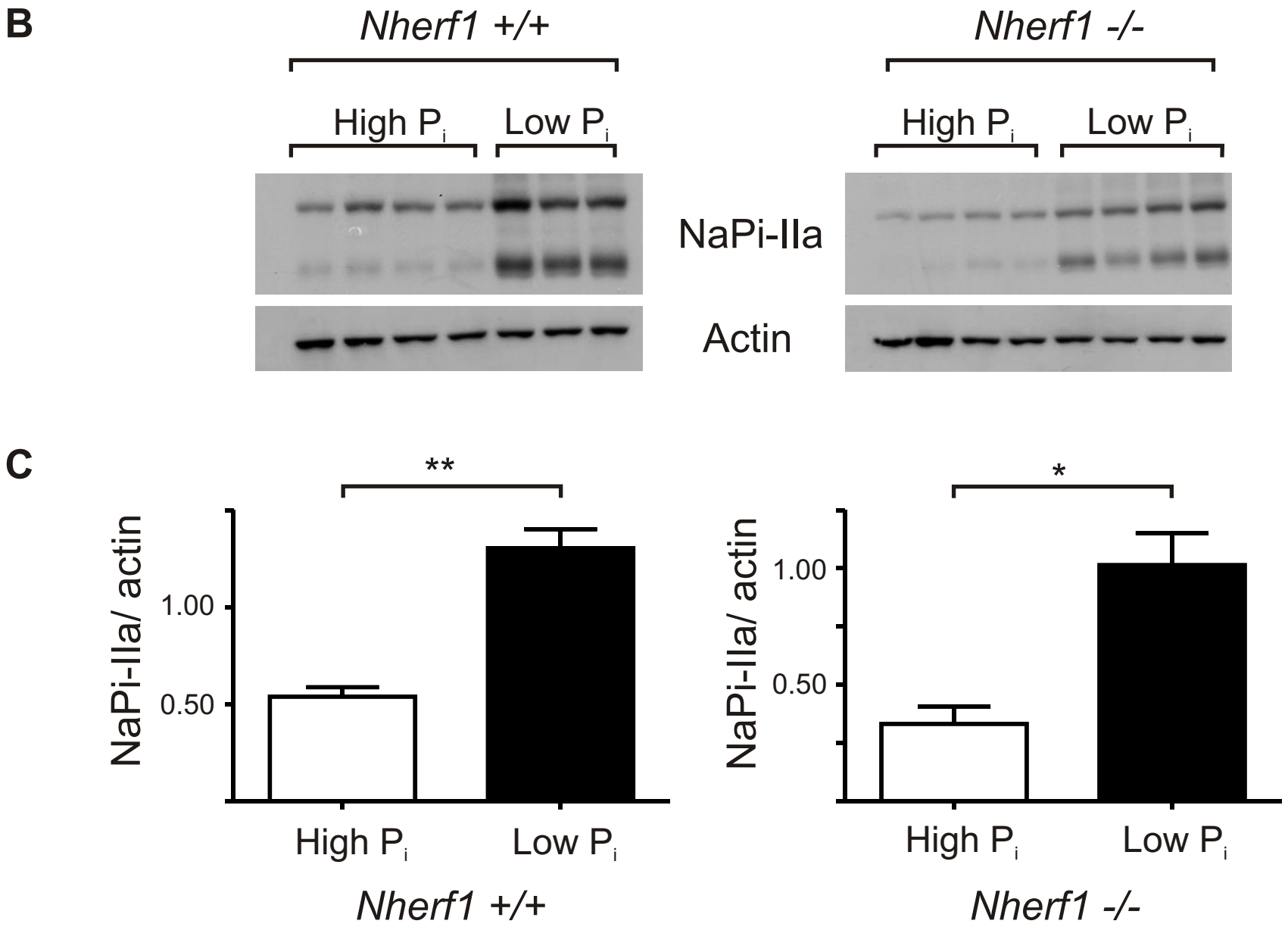


Fig. 6D

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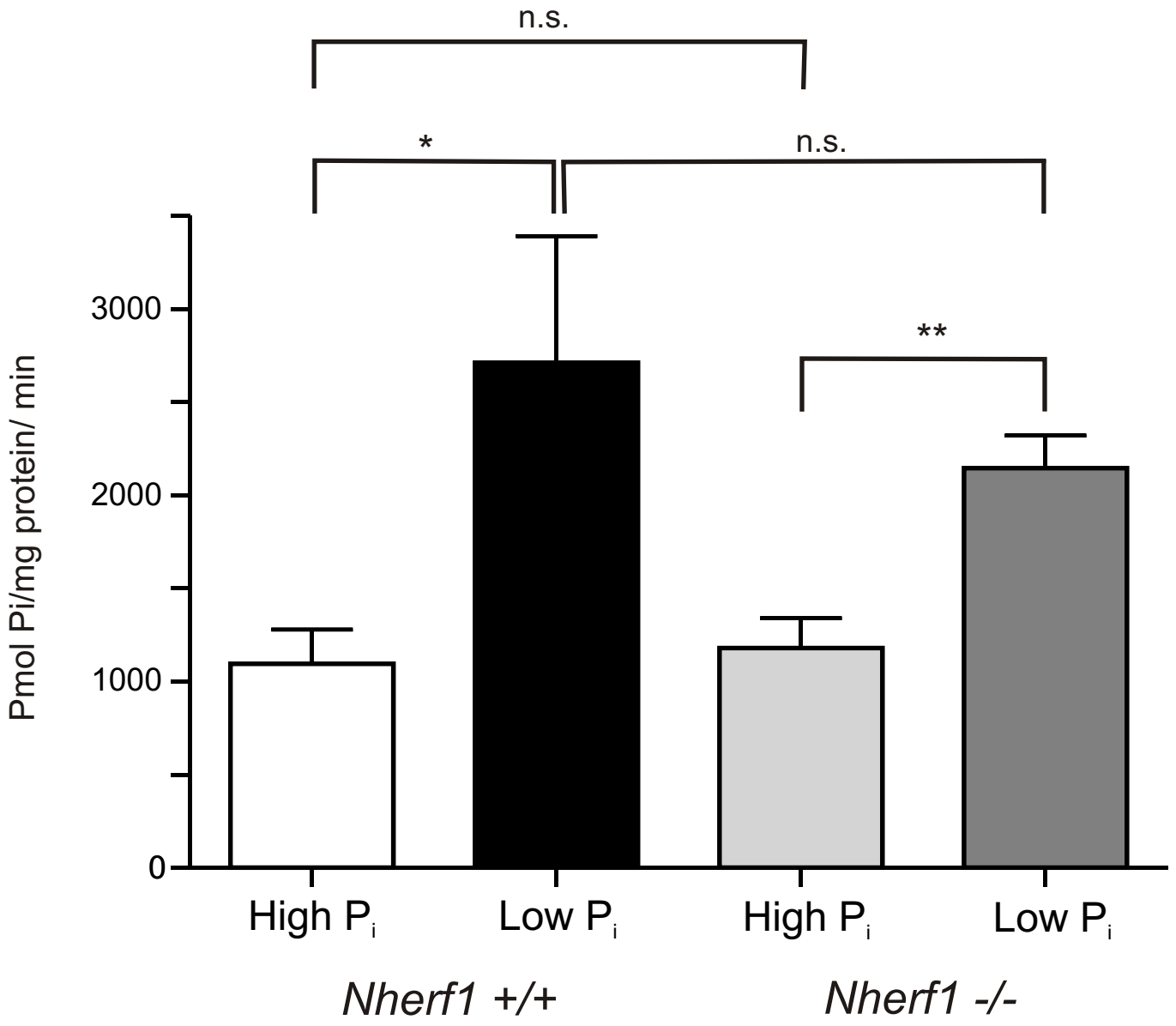

8. SUMMARY OF THE RESULTS

8.1 Role of PDZK1 in NaPi-IIa Regulation

So far little was known about the function of PDZK1 in expression and regulation of NaPi-IIa, due to the fact that no appropriate cell culture model has been available. The recent generation of a *Pdzk1* deficient mouse has made it possible to investigate the role of PDZK1 in NaPi-IIa expression and regulation [22].

Here we investigated urinary phosphate excretion, expression and localization of NaPi-IIa under steady state conditions, demonstrating increased P_i loss in the urine under a high P_i diet in *Pdzk1* deficient mice. Moreover, acute and chronic adaptations of NaPi-IIa to changes in dietary P_i were monitored, finding no difference between control and Knock-Out animals. We did not find any evident difference in the acute PTH-induced down regulation of NaPi-IIa. Finally, we checked for the expression and localization of other PDZK1 interacting proteins than NaPi-IIa, finding that NHERF1/2 abundance increased under high P_i diet conditions.

Taken together these findings suggest that PDZK1 plays no role in the acute regulation of NaPi-IIa either by hormones or dietary changes in P_i intake. However, PDZK1 might be important for trafficking or stability of NaPi-IIa under chronic high P_i intake.

8.2 Role of NHERF1 In NaPi-IIa Regulation

In the *Nherf1* deficient mouse, NaPi-IIa expression in the brush border membrane is reduced and mice present with a mild hyperphosphaturia [42]. Also, adaptation to a low phosphate containing diet or medium is defective in *Nherf1* deficient mice [46] and in a primary proximal tubular cell line derived from *Nherf1* null mice [9]. Here, we were interested in investigating the role of NHERF1 in the regulation of the sodium phosphate cotransporter by PTH, as

it has been recently demonstrated that NHERF1 may be important in transfected cells for the formation of a multi-protein complex allowing for the coupling of the PTH receptor to its downstream effector, PLCβ [28]. We show, in *in vivo* and *in vitro* experiments, that in the proximal tubule NHERF1 is important for a proper PTH induced internalization of NaPi-IIa from the brush border membrane. However, this is not due to a general impairment of the internalization machinery because PTH 1-34, which is acting on both apical and basolateral PTH receptors, was still able to provoke the retrieval of the cotransporter from the membrane; internalization could be directly induced with pharmacological activators of the PKA and PKC pathways; and an acute switch to a high phosphate containing diet still led to internalization and down regulation of NaPi-IIa. Thus, the impairment is specific for the activation of apical PTH receptors. Our experiments demonstrate that the failure to internalize NaPi-IIa in response to PTH 3-34 is caused by the defective coupling of the apical PTH receptor to PLC. Indeed, expression and localization of apical PTH receptors was not affected by the loss of NHERF1, but their ability to increase PLC activity upon stimulation. Thus, NHERF1 is most likely required for the coupling of PLC to apical PTH receptors in the proximal tubule.

8.3 PDZK1, NHERF1, and NaPi-IIa regulation: an integrative view

Trafficking of newly synthesized NaPi-IIa to the apical membrane, anchoring in the brush border membrane, and internalization and transport to the lysosomes require complex interactions with specialized proteins involved in the respective tasks. In an approach aimed to identify such proteins important for the regulation of NaPi-IIa, several PDZ domain containing proteins were found and shown to interact *in vitro* with NaPi-IIa through a classic PDZ-binding motif at its C-terminus. Among these proteins, NHERF1 and PDZK1 are of particular interest, as both proteins have been demonstrated to interact with a variety of membrane proteins such as transporters or receptors and also with several signaling proteins like PKA anchoring proteins (AKAPs) or PLC. In addition, both proteins are able to

interact also with each other. This suggests that NHERF1 and PDZK1 may be part of an apical scaffolding protein network that may be important for positioning of several proteins in close proximity to each other. Such scaffolding may be important for processes like signaling or insertion into and retrieval from the apical membrane.

Based on our data and data form other groups, we can conclude that NHERF1 may be important for the PTH-induced internalization of NaPi-IIa, whereas PDZK1 might serve a stabilizing function which is only unmasked under particular conditions. The fact that, in *Pdzk1 -/-* mice, NHERF1/2 abundance is increased under a high P_i diet where also NaPi-IIa abundance was decreased suggests that NHERF1/2 upregulation might be compensatory but not completely sufficient to prevent the reduction of NaPi-IIa expression.

9. FUTURE PROSPECTS

The small changes in NaPi-IIa expression found in *Pdzk1* deficient mice [8] may be due to compensatory or redundant processes as suggested by the fact that other PDZ proteins such as NHERF1 share many interacting partners with PDZK1. A combined ablation of both NHERF1 and PDZK1 may shed some light on the significance of their overlapping specificities.

As previously shown in our lab, PDZK1 interacts via its four PDZ domains with several proteins expressed in the proximal tubule such as NHERF1, NHE-3, the PKA anchoring protein D-AKAP2, the anion exchanger CFEX/PAT1 (SLC26A6), and the MAP17 protein. To investigate the effect of PDZK1 on the localization and function of some of these interacting proteins may help to elucidate the role of such a complex network of PDZ interactions involved in the polarized expression of membrane proteins in epithelia.

Among the hormones that have been shown to downregulate NaPi-IIa or to induce phosphaturia in vivo, dopamine has also to be mentioned. Using *ex vivo* and *in vitro* techniques, i.e. freshly isolated mouse kidney slices and perfused tubules, we recently demonstrated that luminal D1-like receptors coupling to cAMP/ PKA induce the internalization of NaPi-IIa, reduce its abundance in the brush border membrane, and thus decreases its reabsorptive capacity [1]. As NHERF1 also couples to PKA through ezrin, it would be interesting to investigate the role of this scaffolding protein in the signal transduction triggered by this hormone.

10. PUBLICATIONS THAT DID NOT CONTRIBUTE TO THIS WORK

10.1 Impaired PTH-induced endocytotic down-regulation of the renal type IIa Na+/ P_i-cotransporter in RAP-deficient mice with reduced megalin expression.

Bacic D, Capuano P, Gisler SM, Pribanic S, Christensen EI, Biber J, Loffing J, Kaissling B, Wagner CA, Murer H.

Pflugers Arch. 2003 Jul;446(4):475-84.

Abstract:

Inorganic phosphate (P(i)) reabsorption in the renal proximal tubule occurs mostly via the Na(+)/P(i) cotransporter type IIa (NaP(i)-IIa) located in the brush-border membrane (BBM) and is regulated, among other factors, by dietary P(i) intake and parathyroid hormone (PTH). The PTH-induced inhibition of P(i) reabsorption is mediated by endocytosis of Na/P(i)-IIa from the BBM and subsequent lysosomal degradation. Megalin is involved in receptor-mediated endocytosis of proteins from the urine in the renal proximal tubule. The recently identified receptor-associated protein (RAP) is a novel type of chaperone responsible for the intracellular transport of endocytotic receptors such as megalin. Gene disruption of RAP leads to a decrease of megalin in the BBM and to a disturbed proximal tubular endocytotic machinery. Here we investigated whether the distribution of NaP(i)-IIa and/or its regulation by dietary P(i) intake and PTH is affected in the proximal tubules of RAP-deficient mice as a model for megalin loss. In RAP-deficient mice megalin expression was strongly reduced and restricted to a subapical localization. NaP(i)-IIa protein distribution and abundance in the kidney was not altered. The localization and abundance of the NaP(i)-IIa interacting proteins MAP17, PDZK-1, D-AKAP2, and NHE-RF1 were also normal. Other transport proteins expressed in the BBM such as the Na(+)/H(+) exchanger NHE-3 and the Na(+)/sulphate cotransporter NaSi were normally expressed. In whole animals and in isolated fresh kidney slices the PTH-induced internalization of NaP(i)-IIa was strongly delayed in RAP-deficient mice. PTH

receptor expression in the proximal tubule was not affected by the RAP knock-out. cAMP, cGMP or PKC activators induced internalization which was delayed in RAP-deficient mice. In contrast, both wildtype and RAP-deficient mice were able to adapt to high-, normal, and low-P(i) diets appropriately as indicated by urinary P(i) excretion and NaP(i)-IIa protein abundance.

10.2 Activation of dopamine D1-like receptors induces acute internalization Of The Renal Na+/phosphate cotransporter NaPi-IIa in mouse kidney and OK cells.

Bacic D, Capuano P, Baum M, Zhang J, Stange G, Biber J, Kaissling B, Moe OW, Wagner CA, Murer H.

Am J Physiol Renal Physiol. 2005 Apr;288(4):F740-7

Abstract:

The Na(+)/phosphate cotransporter NaPi-IIa (SLC34A1) is the major transporter mediating the reabsorption of P(i) in the proximal tubule. Expression and activity of NaPi-IIa is regulated by several factors, including parathyroid hormone, dopamine, metabolic acidosis, and dietary P(i) intake. Dopamine induces natriuresis and phosphaturia in vivo, and its actions on several Na(+)-transporting systems such as NHE-3 and Na(+)-K(+)-ATPase have been investigated in detail. Using freshly isolated mouse kidney slices, perfused proximal tubules, and cultured renal epithelial cells, we examined the acute effects of dopamine on NaPi-IIa expression and localization. Incubation of isolated kidney slices with the selective D(1)-like receptor agonists fenoldopam (10 microM) and SKF-38393 (10 microM) for 1 h induced NaPi-IIa internalization and reduced expression of NaPi-IIa in the brush border membrane (BBM). The D(2)-like selective agonist quinpirole (1 microM) had no effect. The D(1) and D(2) agonists did not affect the renal Na(+)/sulfate cotransporter NaSi in the BBM of the proximal tubule. Studies with isolated perfused proximal tubules demonstrated that activation of luminal, but not basolateral, D(1)-like receptors caused NaPi-IIa internalization. In kidney slices, inhibition of PKC (1 microM chelerythrine) or ERK1/2 (20 microM PD-098089) pathways did not prevent the fenoldopam-induced internalization. Inhibition with the PKA blocker H-89 (10 microM) abolished the effect of fenoldopam. Immunoblot demonstrated a reduction of NaPi-IIa protein in BBMs from kidney slices treated with fenoldopam. Incubation of opossum

kidney cells transfected with NaPi-IIa-green fluorescent protein chimera shifted fluorescence from the apical membrane to an intracellular pool. In summary, dopamine induces internalization of NaPi-IIa by activation of luminal D(1)-like receptors, an effect that is mediated by PKA.

10.3 Intestinal and renal adaptation to a low – P_i diet of type II NaPi cotransporters in vitamin D receptor- and 1alphaOHase-deficient mice.

Capuano P, Radanovic T, Wagner CA, Bacic D, Kato S, Uchiyama Y, St-Arnoud R, Murer H, Biber J.

Am J Physiol Cell Physiol. 2005 Feb;288(2):C429-34.

Abstract:

Intake of a low-phosphate diet stimulates transepithelial transport of P_i in small intestine as well as in renal proximal tubules. In both organs, this is paralleled by a change in the abundance of the apically localized NaPi cotransporters NaPi type IIa (NaPi-IIa) and NaPi type IIb (NaPi-IIb), respectively. Low- P_i diet, via stimulation of the activity of the renal 25-hydroxyvitamin-D3-1alpha-hydroxylase (1alphaOHase), leads to an increase in the level of 1,25-dihydroxy-vitamin D3 [1,25(OH)2D]. Regulation of the intestinal absorption of P_i and the abundance of NaPi-IIb by 1,25(OH)2D has been supposed to involve the vitamin D receptor (VDR). In this study, we investigated the adaptation to a low- P_i diet of NaPi-IIb in small intestine as well as NaPi-IIa in kidneys of either VDR- or 1alphaOHase-deficient mice. In both mouse models, upregulation by a low- P_i diet of the NaPi cotransporters NaPi-IIa and NaPi-IIb was normal, i.e., similar to that observed in the wildtypes. Also, in small intestines of VDR- and 1alphaOHase-deficient mice, the same changes in NaPi-IIb mRNA found in wildtype mice were observed. On the basis of the results, we conclude that the regulation of NaPi cotransport in small intestine (via NaPi-IIb) and kidney (via NaPi-IIa) by low dietary intake of P_i cannot be explained by the 1,25(OH)2D-VDR axis.

10.4 PTH treatment induces dissociation of NaPi-IIa/NHERF1 complexes.

Deliot N, Hernando N, Horst-Liu Z, Capuano P, Bacic D, Wagner CA, O'brien S, Biber J, Murer H.

Am J Physiol Cell Physiol. 2005 Mar 23

Abstract:

The type IIa Na/ P_i -cotransporter (NaPi-IIa) and the Na/H-exchanger regulatory factor 1 (NHERF1) colocalize in the apical membrane of proximal tubular cells. Both proteins interact in vitro. Here, the interaction between NaPi-IIa and NHERF1 is further documented by coimmunoprecipitation and copulldown assays. NaPi-IIa is endocytosed and degraded in lysosomes upon parathyroid hormone (PTH) treatment. To investigate the effect of PTH on the NaPi-IIa/NHERF1 association, we first compared the localization of both proteins after PTH treatment. In mouse proximal tubules and OK cells NaPi-IIa was removed from the apical membrane after hormonal treatment; however, NHERF1 remained at the membrane. Moreover, PTH led to degradation of NaPi-IIa, without changes in the amount of NHERF1. The effect of PTH on the NaPi-IIa/NHERF1 interaction was further studied by coimmunoprecipitation. PTH treatment reduced the amount of NaPi-IIa coimmunoprecipitated with NHERF antibodies. PTH-induced internalization of NaPi-IIa requires PKA and PKC; therefore we next analyzed whether PTH induces changes in the phosphorylation state of either partner. NHERF1 was constitutively phosphorylated. Moreover, in mouse kidney slices PTH induced an increase of NHERF1 phosphorylation; independent activation of PKA or PKC also resulted in an increased phosphorylation of NHERF1 in kidney slices. However, NaPi-IIa was not phosphorylated either basally or after exposure to PTH. In summary, our study supports an interaction between NHERF1 and NaPi-IIa based on their brush border membrane colocalization and in vitro coimmunoprecipitation/copulldowns. Furthermore, PTH weakens

this interaction, as evidenced by different in situ/in vivo behavior. The PTH effect takes place in the presence of increased phosphorylation of NHERF1.

10.5 NaPi-IIa and interacting proteins.

Hernando N, Gisler SM, Pribanic S, Deliot N, Capuano P, Wagner CA, Moe OW, Biber J, Murer H.

J Physiol. 2005 May 12

Abstract:

Regulation of renal proximal tubular reabsorption of phosphate (P_i) is one of the critical steps in P_i homeostasis. Experimental evidences suggest that this regulation is achieved mainly by controlling the apical expression of the type-IIa Na-dependent P_i-cotransporter (NaPi-IIa) in proximal tubules. Only recently have we started to obtain information regarding the molecular mechanisms that control the apical expression of NaPi-IIa. A first critical observation was the finding that truncation of only its last three amino acid residues has a strong effect on apical expression. A second major finding was the observation that the last intracellular loop of NaPi-IIa contains sequence information that confers PTH sensitivity. The use of the above domains of the cotransporter in yeast two-hybrid (Y2H) screen allowed the identification of proteins interacting with NaPi-IIa. Biochemical, morphological as well as functional analyses have allowed us to obtain insights into the physiological roles of such interactions, though our present knowledge is still far from complete.

10.6 Regulation Of Sodium-Proton Exchanger NHE-3 By PKA And EPAC

Honegger K, Capuano P, Winter C, Bacic D, Stange G, Wagner CA, Biber J, Murer H and Hernando N

Proc Natl Acad Sci U S A. 2006 Jan 17;103(3):803-8

Abstract:

The Na/H-exchanger 3 (NHE-3) is expressed in the brush border membrane (BBM) of proximal tubules. Its activity is downregulated upon increases in intracellular cAMP levels. The aim of this study was to investigate the contribution of the protein kinase A- (PKA) and the exchange protein directly activated by cAMP- (EPAC) dependent pathways in the regulation of NHE-3 by cAMP.

OK cells and murine kidney slices were treated with cAMP analogs which selectively activate either PKA or EPAC. Activation of either pathway resulted in an inhibition of NHE-3 activity. The EPAC-induced effect was independent of PKA as indicated by the insensitivity to the PKA-inhibitor H89. Both PKA and EPAC inhibited NHE-3 activity without inducing changes in the expression of the transporter in BBM. In contrast, activation of PKA but not of EPAC inhibited NaPi-IIa, other Na-dependent transporter expressed in proximal BBM. PKA but not EPAC induced the retrieval of NaPi-IIa from BBM.

Our results suggest that EPAC activation may represent a novel mechanism involved in the cAMP regulation of NHE-3, whereas regulation of NaPi-IIa is mediated by PKA but not by EPAC.

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